Food Research

An Official Publication of

The Institute of Food Technologists

IN THIS ISSUE

Cake Structure and Palatability as Affected by Emulsifying Agents and Baking Temperatures

Some Aspects of Microbiology Applied to Commercial Apple Juice Production. Part IV. Development Characteristics and Viability of Spoilage Organisms in Apple Juice

Some Aspects of Microbiology Applied to Commercial Apple Juice Production. Part V. Thermal Death Rates of Spoilage Organisms in Apple Juice

A Comparison of Two Methods for Determining The Collagen Content of Cooked Meat

The Effect of Ingredient Variations on the Quality of White and Chocolate Cakes Baked Prior to Freezing or Baked From Frozen Batters

Reduction of Trimethylamine Oxide in Dogfish Flesh

Exploratory Experiments to Identify Chemical Reactions Causing Flavor Deterioration During Storage of Canned Orange Juice. I. Incompatibility of Peel-Oil Constituents With the Acid Juice

Retention of Nutrients in Commercially Prepared Soybean Curd

A Study of the Control of the Sanitary Quality of Custard-Filled Bakery Products in a Large City

A Study of the Effect of Various Substances on the Volatility of Diacetyl at an Elevated Temperature

Acid-Tolerant Bacteria in Citrus Juices

The Kinetics of the Oxidation of Strawberry Anthocyanin by Hydrogen Peroxide

Factors Which Affect the Drained Weight and Other Characteristics of Heat-Processed Red Cherries

Volume 17 MAY-JUNE, 1952 Number 3

Food Research

An Official Publication of
The Institute of Food Technologists

Volume 17 MAY-JUNE, 1952 Number 3

EDITORIAL BOARD

Z. I. KERTESZ, *Editor-in-Chief*
New York State Agricultural Experiment Station
Geneva, New York

A. K. BALLS
J. S. BLAIR
G. M. DACK
ROBERT S. HARRIS
L. B. HOWARD
L. B. JENSEN
M. A. JOSLYN
A. G. LOCHHEAD
PAULINE PAUL
CATHERINE J. PERSONIUS
SAMUEL C. PRESCOTT
J. J. WILLAMAN

FRED W. TANNER, *Editor Emeritus*

CHARLES S. LAWRENCE, *Business Manager*
176 West Adams Street
Chicago 3, Illinois

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 119-123 West Park Avenue, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879.

the ROCHE REVIEW *of enrichment requirements*

PRODUCT	Thiamine (B_1)		Riboflavin (B_2)		Niacin		Iron	
	MIN.	MAX.	MIN.	MAX.	MIN.	MAX.	MIN.	MAX.
Enriched BREAD, or other baked products	1.1	1.8	0.7	1.6	10.0	15.0	8.0	12.5
Enriched FLOUR[1]	2.0	2.5	1.2	1.5	16.0	20.0	13.0	16.5
Enriched FARINA[2]	1.66	—	1.2	—	6.0	—	6.0	—
Enriched MACARONI products[3]	4.0	5.0	1.7	2.2	27.0	34.0	13.0	16.5
Enriched NOODLE products[3]	4.0	5.0	1.7	2.2	27.0	34.0	13.0	16.5
Enriched CORN MEALS	2.0	3.0	1.2	1.8	16.0	24.0	13.0	26.0
Enriched CORN GRITS[4]	2.0	3.0	1.2	1.8	16.0	24.0	13.0	26.0
Enriched MILLED WHITE RICE[5]	2.0	—	*		16.0	—	13.0	—

ALL FIGURES REPRESENT MILLIGRAMS PER POUND

1. In enriched self-rising flour, calcium is also required between limits of 500-1500 mg. per pound. 2. No maximum levels have been enforced. 3. Levels allow for 20-50% losses in kitchen procedure. 4. Levels must not fall below 85% of minimum figures after a specific rinsing test described in the Federal Standards of Identity. 5. Levels must not fall below 85% of levels shown after washing and rinsing. *Omitted in the U.S.A. and Puerto Rico but used in certain Far Eastern countries to a minimum level of 1.2 mg. per pound.

The maximum and minimum levels shown above for enriched bread, enriched flour, enriched farina, enriched macaroni, spaghetti and noodle products, enriched corn meal and corn grits are in accordance with Federal Standards of Identity or State laws. Act No. 183 of the Government of Puerto Rico requires the use of enriched flour for all products made wholly or in part of flour. This includes crackers, pretzels, etc.

The levels for milled white rice are officially those of the Government of Puerto Rico. These levels are commonly accepted in U. S. domestic and export marketing and are based on the recommendation of the Committee on Cereals, Food and Nutrition Board, National Research Council.

ROCHE **VITAMINS FOR ENRICHMENT**

HERE'S HELP FOR YOU. We have reprinted the information in this advertisement on a handy 3" x 5" book-mark card. If you wish a quantity of this helpful resume for distribution to others in your organization, in teaching, or for your own files, please send your name and address to the Vitamin Division, stating the number you need.

VITAMIN DIVISION · HOFFMANN-LA ROCHE INC. · NUTLEY 10, N. J.

In Canada: Hoffmann-La Roche, Ltd., Montreal, Que.

Volume 17 May-June, 1952 Number 3

CONTENTS

Cake Structure and Palatability as Affected by Emulsifying Agents and Baking Temperatures. Martha E. Jooste and Andrea Overman Mackey . . 185

Some Aspects of Microbiology Applied to Commercial Apple Juice Production. Part IV. Development Characteristics and Viability of Spoilage Organisms in apple juice. C. R. Marshall and V. T. Walkley 197

Some Aspects of Microbiology Applied to Commercial Apple Juice Production. Part V. Thermal Death Rates of Spoilage Organisms in Apple Juice. C. R. Marshall and V. T. Walkley 204

A Comparison of Two Methods for Determining the Collagen Content of Cooked Meat. Ruth M. Griswold and Florence Leffler 212

The Effect of Ingredient Variations on the Quality of White and Chocolate Cakes Baked Prior to Freezing or Baked From Frozen Batters. Andrea Overman Mackey, Patsy Jones, and Joyce Dunn 216

Reduction of Trimethylamine Oxide in Dogfish Flesh. R. Paul Elliott . . . 225

Exploratory Experiments to Identify Chemical Reactions Causing Flavor Deterioration During Storage of Canned Orange Juice. I. Incompatibility of Peel-Oil Constituents With the Acid Juice. J. S. Blair, Edith M. Godar, J. E. Masters, and D. W. Riester 235

Retention of Nutrients in Commercially Prepared Soybean Curd. Carey D. Miller, Helen Denning, and Adelia Bauer 261

A Study of the Control of the Sanitary Quality of Custard-Filled Bakery Products in a Large City. Abraham E. Abrahamson, Rubin Field, Leon Buchbinder, and Anna V. Catelli 268

A Study of the Effect of Various Substances on the Volatility of Diacetyl at an Elevated Temperature. Anne Conrad Stoll 278

Acid-Tolerant Bacteria in Citrus Juices. L. W. Faville and E. C. Hill . . . 281

The Kinetics of the Oxidation of Strawberry Anthocyanin by Hydrogen Peroxide. Ernest Sondheimer and Z. I. Kertesz 288

Factors Which Affect the Drained Weight and Other Characteristics of Heat-Processed Red Cherries. R. T. Whittenberger 299

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $4.50 per volume.

Non-members: Domestic, $5.50 per volume; foreign, $6.00 per volume.

Subscriptions and renewals are entered to begin with the first issue of the current volume.

Single copies of current volume: Domestic, $1.00; foreign, $1.15.

CORRESPONDENCE

Editorial: Z. I. Kertesz, Editor-in-Chief, New York State Agricultural Experiment Station, Geneva, New York.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: Food Research, 119-123 W. Park Ave., Champaign, Ill.

Members Subscriptions: The Institute of Food Technologists, 176 W. Adams St., Chicago 3, Ill.

Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in Food Research. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for Food Research should conform to the style used in the journal and be submitted in duplicate to the Editor-in-Chief. Authors of papers intended for Food Research should request from the Editorial Office a copy of the booklet "Information and suggestions for authors." Food Research reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

Copyright, 1952
By the Institute of Food Technologists
Printed in U. S. A.

NEW EDITION OF "INFORMATION AND SUGGESTIONS FOR AUTHORS" PUBLISHED

A new and expanded edition of the pamphlet "Information and suggestions for authors" has been published for those writing technical contributions for *Food Technology* and *Food Research*.

The pamphlet discusses the respective scopes of the two journals and the type of articles suitable for them. A detailed outline is presented of the customary organization of technical papers followed by statements concerning the preferred styles and methods of preparing manuscripts. A discussion of various details like units of measurements and their abbreviations, use of footnotes and literature citations, preparation of tables and figures, etc. follows.

In the following chapter the customary methods of correcting printer's proofs are described. Here a list is given indicating the more common corrections. Instructions for the ordering of reprints follow.

Prospective authors are urged to consult this pamphlet which will be sent free of charge upon a request addressed to the editorial office.

Authorship in *Food Technology* and *Food Research* is not restricted to members of the Institute of Food Technologists.

Food Technology

Published Monthly by the

INSTITUTE OF FOOD TECHNOLOGISTS

The Companion Journal to

FOOD RESEARCH

If you are a reader of FOOD RESEARCH your library is incomplete without FOOD TECHNOLOGY—devoted to the publication of the results of research and their practical application in industry. Contributors to its pages represent many fields of scientific and technological interest.

SUBSCRIPTION RATES

FOOD RESEARCH	$ 5.50 Domestic	$ 6.00 Foreign
FOOD TECHNOLOGY . . .	7.50 Domestic	8.50 Foreign
Both Journals	12.00 Domestic	13.50 Foreign

Send Subscriptions to

FOOD TECHNOLOGY
119-123 West Park Avenue
CHAMPAIGN, ILLINOIS

You Asked for SPEED . . . for ACCURACY . . . Choice of TOTAL SOLIDS, INDEX SCALES and BUTYRO . . .

You Get *All Three* in the

WORLD'S FASTEST REFRACTOMETER

Featuring

"STOP-WATCH" SPEED!

± .0001 **"LABORATORY" ACCURACY on the PRODUCTION LINE!**

- ***Super Speed Readings*** . . . from ALL NEW DESIGN! Want analyses in 10 seconds? 15 seconds? You can have the speed your process requires. *No more losses of time, materials and money due to slow readings!* This new Bausch & Lomb *Industrial* Refractometer is strictly a *production* instrument . . . designed and built for use right beside your vats. You'll want several . . . at least one for *each* testing area.
- ***Super Speed Loading*** . . . new prism design.
- ***Super Speed Cleaning*** . . . prisms easily, quickly cleaned. Prevents contamination of new samples. Internal parts protected from dirt, fumes, stray liquids.
- ***Super Temperature Stability*** . . . easier to maintain stable temperatures.

Plus dependable, uniform accuracy of ±.0001. Simplified to basic essentials . . . operated by any average worker. Scales "tailor-made" to seven specific ranges industry requires.

New Bausch & Lomb *Industrial* REFRACTOMETER

SEND TODAY FOR COMPLETE DETAILS

BAUSCH & LOMB OPTICAL COMPANY
76506 ST. PAUL STREET, ROCHESTER 2, NEW YORK

Please send me complete information on the New Bausch & Lomb Industrial Refractometer.

NAME_____

TITLE_____

COMPANY_____

STREET_____

CITY_____ ZONE____ STATE_____

CAKE STRUCTURE AND PALATABILITY AS AFFECTED BY EMULSIFYING AGENTS AND BAKING TEMPERATURES[a]

MARTHA E. JOOSTE[b] AND ANDREA OVERMAN MACKEY
Department of Home Economics, Agricultural Experiment Station, Oregon State College, Corvallis, Oregon

(Manuscript received June 9, 1951)

Microscopic studies of cake batters have shown that they are 2-phase systems, one phase being the fat, and the second phase consisting of all the other ingredients of the mixture *(1)*. Hence, a cake batter containing fat and eggs has been referred to as a complex food emulsion *(5)*.

The recognition of the emulsion nature of cake batters has stimulated efforts to improve batter structure and cake quality through the use of emulsifying agents.

When small percentages of certain emulsifying agents such as mono- and diglycerides of fatty acids are added to high sugar ratio cakes, a finer grain, improved effect on tactile senses, and increased volume are obtained *(4)*. These effects have been ascribed to the increased dispersion of fat in the presence of emulsifier *(5)*.

Since air occluded during mixing appears for the most part in the fat *(3)* the fine dispersion pattern of the shortening might be expected to result in an increased dispersion of air. This along with the finely divided fat may account for the effectiveness of the emulsifier.

Cake batters containing monoglycerides have been said to show a marked resistance to variable conditions of baking and handling *(4)*. If this is the case, impaired cake structure which results when low oven temperatures are used *(7)*, might possibly be corrected by the use of such stabilizers.

The purposes of this study were: 1, to determine the effect of glyceryl monostearate on the structure and palatability of cakes made with 2 fats and baked at various oven temperatures; and 2, to obtain information as to the function of the emulsifier, through a study of fat and air distribution in batters and baked cakes.

EXPERIMENTAL PROCEDURE

For these experiments 2 types of fat, butter and hydrogenated vegetable oil, with and without added emulsifier were tested in cakes baked at 4 different temperatures, 300°, 325°, 375°, and 425°F.(149°, 163°, 190.5°, and 218.5°C.). The emulsifier, glyceryl monostearate, was prepared for use as a water dispersion and as a fat dispersion, each being used at 3 and 6% levels (fat basis).

Experimental plan. The formula, containing a high ratio of sugar to flour, and the method of mixing employed had been developed for studies of fat behavior in cake *(8)*. When an emulsifier was used, adjustments were made in both liquid and fat to maintain both at the same level as in the original formula. A fat dispersal of glyceryl monostearate was obtained by melting it with ¼ of the weighed fat, then

[a] Published as Technical Paper No. 694, with the approval of the Director of the Oregon Agricultural Experiment Station.

[b] Permanent address: Department of Home Economics, University of Stellenbosch, Stellenbosch, Union of South Africa.

quickly mixing it with the remaining fat. The water dispersion was obtained by heating 7 parts of water with 3 parts of glyceryl monostearate. This was weighed out as required. All techniques were standardized before beginning the experimental work herewith reported.

The experimental design was a $4 \times 2 \times 5$ factorial in randomized blocks with 3 replications. Altogether 120 cakes were prepared for scoring and 120 for the determination of volumes and for microscopic study.

Immediately after the batters were finished, 2 portions were weighed into pans and baked. While the cakes were baking, slides were prepared for microscopic study of batter structure, and the specific gravity of the batters was determined. For this purpose an aluminum pycnometer designed for the determination of the specific gravity of semifluid materials was used. The pycnometer consisted of 3 parts: a cup, a lid with a hole 3 mm. in diameter, and a threaded rim for screwing the lid onto the cup. The batter was placed carefully in the cup and the lid screwed down tightly. Excess batter was forced out through the hole in the lid and removed. Specific gravity was found by comparing the weight of the fixed volume of batter with that of the same volume of water, both weighed at 25°C.

The time necessary for baking cakes at each oven temperature was established through observation of internal temperature of the cakes, appearance of the crust and shrinkage during baking.

Data were collected on baked cakes as follows: weight loss during baking, cross section areas measured by means of a compensating polar planimeter and used as an index to volume, subjective scores, and slides and photomicrographs for studies of structure.

Microscopic studies. A minute portion of batter was transferred to a glass slide and spread out with the aid of a cover glass and forceps to obtain a thin smear. For better differentiation of fat, smears were also made of batters stained with Sudan IV, a fat soluble dye.

A study of ingredients' distribution in cakes necessitated fixing and staining of the fat with osmic acid. After the fat was fixed, the cake crumb could be cleared and embedded in wax for microsectioning. Deparaffinized sections were stained with eosin and iodine in order to demonstrate protein and starch distribution *(6)*.

Scoring. The cakes were scored the same day they were baked, by 3 experienced judges. A control cake was prepared each day to supply a standard with which the experimental cakes could be compared. For the control, the formula for the basic cake was used, with butter as fat and 3% water-dispersed glyceryl monostearate. It was baked at 375°F.(190.5°C.).

The judges were asked to score 10 different characteristics with reference to those of the control cake which were assigned a score of 5. When a characteristic appeared to be better in a test cake than in the control, a score higher than 5 was given, and when the characteristic was poorer, the scores went down. The range of scoring was from 1 to 10.

Statistical analysis. Analyses of variance were used for batter specific gravity, cake scores, and cake volumes, in testing the significance of differences among emulsifying agents and baking temperatures, in order to determine the merit of the different treatments. The 5% significance level was used throughout.

RESULTS AND DISCUSSION

Batters

Glyceryl monostearate and foam formation during creaming. "Creaming," the formation of an air-in-fat foam *(7)* was the first step in the mixing of the cakes. Both butter and hydrogenated vegetable oil exhibited good creaming properties as shown by the development of lightness and fluffiness during mixing of the fat and sugar. The creaming properties of both fats were impaired in the presence of emulsifier. When an emulsifier was added to a mix that had already been creamed to a light, fluffy consistency, air was immediately lost, and the mixture became pasty and compact.

The specific gravity of the creamed mixture of butter and sugar without emulsifying agents was 1.013 and that of the creamed mixture plus 6% glyceryl monostearate was 1.305. The hydrogenated vegetable shortening always incorporated more air than butter, so that the specific gravity of the creamed mixture was 0.937 without emulsifier and 1.187 when 6% glyceryl monostearate was present.

Glyceryl monostearate and the physical structure of the batter. The batters made with butter and with hydrogenated vegetable oil in the absence of an emulsifying agent were well aerated, light and thick, resembling whipped cream. Characteristically, the batters showed a tendency to break. When 6% emulsifying agent was present, the batters were smooth, glossy, relatively thick and showed a slight tendency to flow, but no break or separation occurred. The batters lacked the lightness of batters without emulsifying agent and appeared more like medium thick white sauce than like whipped cream. Batters containing 3% glyceryl monostearate were between these 2 extremes in smoothness and lightness.

When the batters were studied microscopically, the most obvious difference between those made with and without emulsifier was in the fat and air distribution. When no emulsifying agent was present, the fat together with occluded air bubbles appeared in clumps, whereas the fat and air became more finely dispersed as the percentage of emulsifier was increased.

The dispersion of fat and gas bubbles was different in batters made with the 2 fats. The hydrogenated vegetable oil batters with and without emulsifier always showed a greater number of gas bubbles than the corresponding butter batters. It will be recalled that the hydrogenated shortening also produced lighter creamed mixes than butter. Butter plus emulsifier, on the other hand, was more highly dispersed than the vegetable shortening plus emulsifier. Hence the occluded air bubbles were more uniformly distributed.

The fat-dispersed emulsifier as compared with the water-dispersed, gave a better distribution of fat.

The types of dispersion of fat and air bubbles in batters made with the two fats, with and without emulsifier, are shown in Figures 1, 2, 3, and 4. The fat appears gray, air bubbles are round and sharply defined, and the rest of the ingredients appear light gray or white. In the picture showing butter plus emulsifier, the fat was uniformly dispersed in such thin films that it cannot be distinguished from other ingredients in a black and white picture.

Volume of Baked Cakes

The analysis of variance of cake volumes is shown in Table 2. As has been mentioned, the experimental design was a $4 \times 2 \times 5$ factorial in randomized blocks with 3 replications. Thus, 120 cakes were baked for the determination of volume, and 6 observations were made on each cake. The experimental error was the variation among the cakes which received the same treatment. The sampling error was the variation among the observations made on the same cake. The effect of replication (block) was not significant, hence the sum of squares for replication was pooled with that of experimental error.

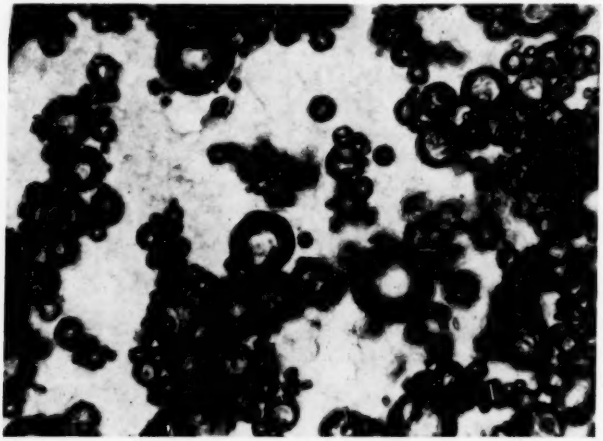


Figure 1. Photomicrograph (120×) of cake batter made with butter. Fat (gray) appears in clumps as indicated by the irregular grouping of gas bubbles. The spheres with the black outlines are the gas bubbles. The rest of the ingredients appear white.

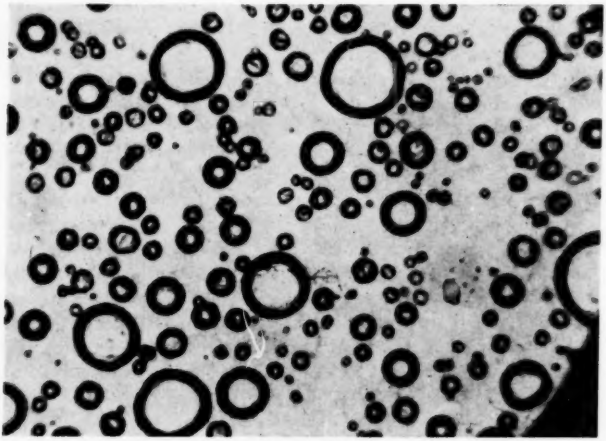


Figure 2. Photomicrograph (120×) of cake batter made with butter plus 6% emulsifier. Fat is so well dispersed that there is little contrast between fat and the rest of the ingredients. Gas bubbles are evenly distributed.

Emulsifying agent. As would be expected, the difference in batter structure was reflected in the cakes baked of these batters. When emulsifier was added, cake volume increased, and cell structure was improved. However, increasing the emulsifier from 3 to 6% did not significantly improve cake volume except at the highest baking temperature. Cell structure will be discussed in a later paragraph.

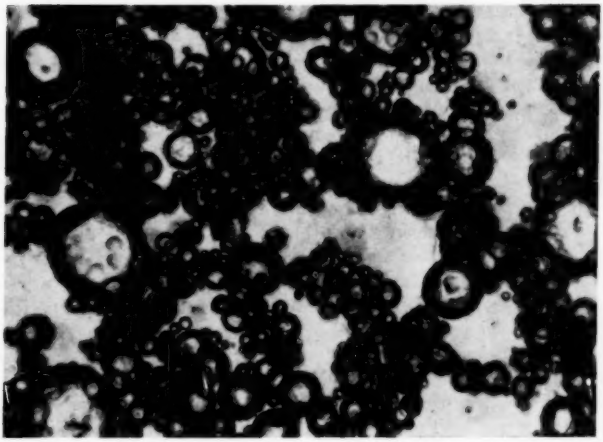


Figure 3. Photomicrograph (120×) of cake batter made with hydrogenated vegetable shortening. A well-aerated batter, with large numbers of gas bubbles crowded together in clumps of fat.

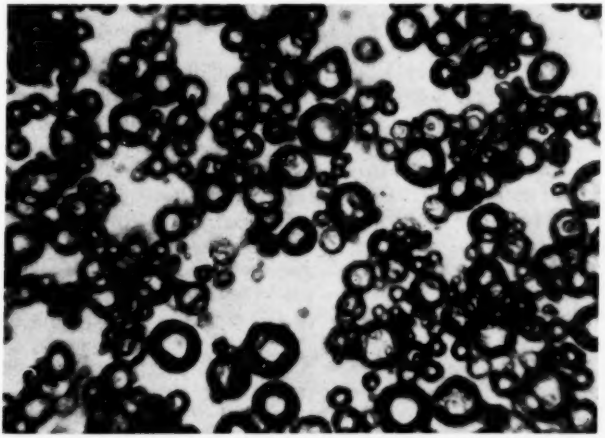


Figure 4. Photomicrograph (120×) of cake batter with hydrogenated vegetable shortening, plus 6% emulsifier. Fat and gas bubbles are well dispersed throughout the batter.

The larger volumes obtained when emulsifier was present indicated that more gas was retained in the batter at the time that coagulation of the protein occurred, or that the leavening action of occluded gas was enhanced due to improved structural characteristics of the surrounding films. The emulsifier present in the cake batter could orient itself at the air-liquid interface causing a variation in the surface forces of the liquid in relation

to the gas. In this way it would assist in stabilizing the foam in the cake batter.

During the baking, cells would not coalesce as readily in emulsifier-containing cakes. The emulsifier-reinforced films surrounding the gas bubbles in these cakes would possess greater mechanical strength and greater elasticity. Under pressure of expanding gas they would tend to stretch but not break. This is evidenced by the larger volumes as well as improved cell structure shown by emulsifier-containing cakes.

In the previous paragraphs it has been assumed that the increased cake volume could be ascribed to a better pattern of gas distribution and surface film characteristics. Other possible explanations for the increased volumes considered and experimentally disproved were: 1, whether increased volume could have been due to increased moisture retention; and 2, whether volume increase could be accounted for by the incorporation of more air in the mixed batters.

Cake quality appears to be affected by all the ingredients incorporated. If moisture retention during baking influences cake volume or palatability, the presence of glyceryl monostearate might affect these qualities indirectly through its hydrophilic properties.

Moisture loss was determined by the weight of the batter minus the weight of the baked cake. The loss in weight during baking may be considered largely due to evaporation *(2)*. Little browning of the crust took place at the lower baking temperatures, hence the greater loss in moisture at these temperatures was not due to caramelization of sugars in the crust.

The loss in weight during baking was found to be related to baking time, but not to cake volume, nor to emulsifier treatment. Cake volume was not, therefore, related to moisture retention during baking, nor was moisture retention affected by the incorporation of glyceryl monostearate in the cake batter. On the other hand, the loss in weight of cakes during baking was related to palatability scores. The cakes that had been baked for a longer time and had lost the most weight were scored lower for the palatability factors of moistness, springiness, and velvetiness.

The second possibility, namely, that the increased volume may be due to increased aeration of the batters was investigated through specific gravity measurements on the batters. Specific gravity was determined on 120 different batters. The average specific gravity for each treatment may be compared with cake volumes, Table 1.

If the supposition is correct that better aeration of batters produces bigger cake volume, the largest cake should have been obtained when hydrogenated vegetable oil was used without emulsifier. Instead it was found that better cake volumes were obtained from the less aerated batters.

Since the increased volume could not be correlated with the incorporation of air, or moisture retention, the postulation that better gas retention during the latter stages of baking accompanied with greater elasticity of air bubble surfaces was responsible for the volume obtained seemed most tenable. Even though less air was present in the batter containing an emulsifier, the air must have been used to better advantage.

Fat and baking temperature. The analysis of variance of cake volumes, Table 2, not only showed a significant difference due to the addition of

TABLE 1

Average specific gravities of batters and average cross section areas of cakes prepared with 2 fats, with and without glyceryl monostearate (GMS) emulsifier, and baked at 4 temperatures

Baking temperature		Butter					Vegetable fat				
		No emulsifier	GMS, fat-dispersed		GMS, water-dispersed		No emulsifier	GMS, fat-dispersed		GMS, water-dispersed	
			3%	6%	3%	6%		3%	6%	3%	6%
		Specific gravity of batters									
°F.	°C.	0.780	0.840	0.827	0.856	0.856	0.709	0.775	0.820	0.813	0.841
		Cross section area of cakes in square inches									
425	218.3	4.30	5.40	5.68	5.46	5.63	4.57	4.79	5.32	5.37	5.61
375	190.6	4.33	4.93	5.48	5.09	5.34	4.44	4.57	4.76	4.84	5.09
325	163.2	4.63	4.74	4.89	4.82	4.71	4.20	4.57	4.61	4.59	4.69
300	148.9	4.11	3.92	4.18	4.09	4.25	4.09	3.78	3.95	4.09	3.83

emulsifiers, but also due to fat and to baking temperature used. Butter cakes which contained emulsifier were larger than corresponding cakes made with vegetable shortening. This may be accounted for by the greater dispersion of butter accompanied with a more uniform distribution of gas bubbles through the batter. The greater dispersion of butter may be due in part to the reinforcing action of emulsifiers normally present in butter.

The volumes of emulsifier cakes increased with each increase in baking temperature. When no emulsifier was added, cake volumes were approximately the same regardless of the temperature at which they were baked, Table 1.

TABLE 2

Analysis of variance of cake volumes

Source of variation	Degrees of freedom	Mean squares
Temperatures	3	45.1237*
Fats	1	8.0412*
Cakes	4	7.8611*
No emulsifier vs. emulsifier	1	23.3388*
Among emulsifiers	3	8.1057
Temperatures × fats	3	0.1928
Temperatures × cakes	12	1.7544*
Fats × cakes	4	0.7966
Temperatures × fats × cakes	12	0.4952*
Experimental error	80	0.1268
Sampling error	600	0.0033
Total	719	.....

*Indicates significance at 5% level.

The small volumes of cakes baked at low temperatures may be due to excessive loss of gas and coalescence of cells because of delayed coagulation of egg and flour proteins *(7)*. That this was probably the case is suggested by the fact that the internal temperature of cakes baked at 300°F. and 325°F. rose more slowly and reached a lower maximum than did the internal temperature of cakes baked in hotter ovens, Table 3.

TABLE 3

Maximum internal temperature, and time required to bake cakes at 4 oven temperatures

Baking temperature		Maximum internal temperature		Baking time
°F.	°C.	°F.	°C.	*minutes*
425	218.3	230	110	13.5
375	190.6	230	110	19.0
325	163.2	221	105	33.0
300	148.9	221	105	62.0

External appearance. The appearance of the cakes was influenced by the shape of the top and by the crust characteristics.

The tops of cakes baked at 325° and 375°F.(163° and 190.5°C.) were level in the absence of emulsifier but rounded when an emulsifier was present. When baked at 425°F.(218.5°C.) cakes tended to peak and occasionally a small center crack developed. When baked at 300°F.(149°C.) the tops of cakes tended to sink. Cross sections of cakes are shown in Figures 5 and 6.

Crust characteristics. The quality of the crust was influenced by the degree of brownness, adherence of the crust to the sides of the pan, the presence of white dots, gumminess, and smoothness.

Brownness and adherence of crust to the sides of the pan were affected by baking temperature but not by the presence of emulsifier nor by the

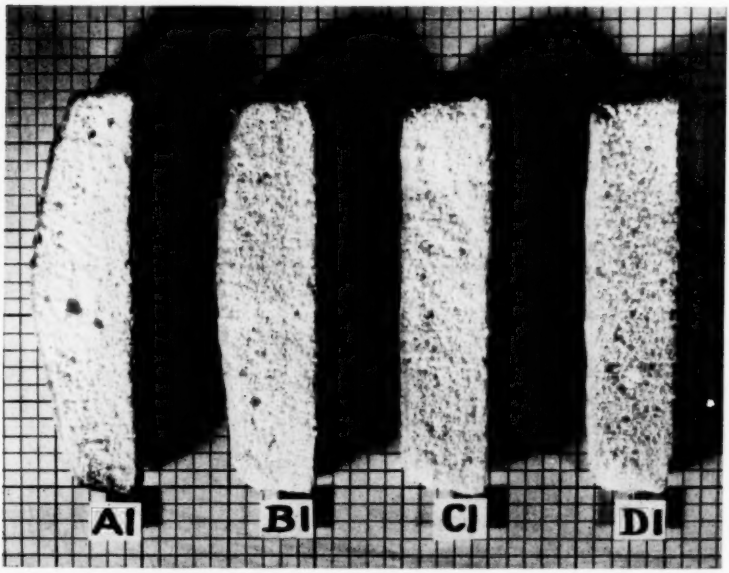


Figure 5. Butter cakes made without emulsifier. Cake A1 was baked at 425°F.; B1, at 375°F.; C1, at 325°F.; and D1, at 300°F.

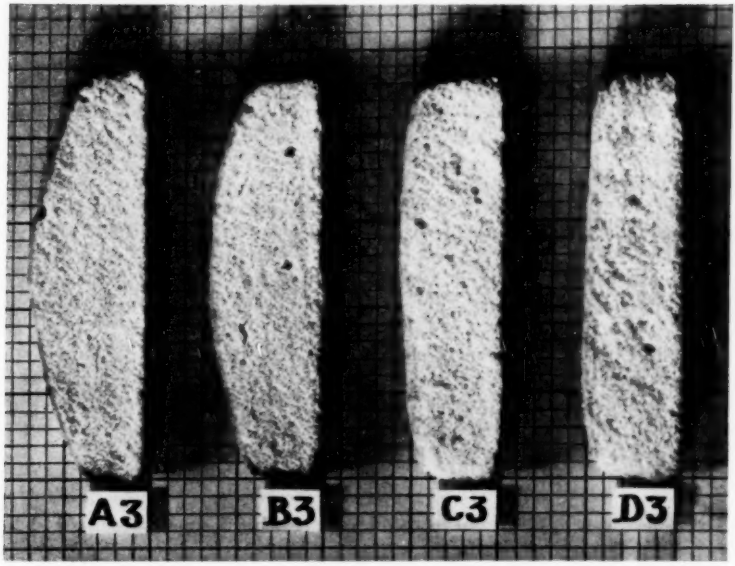


Figure 6. Butter cakes made with 6% glyceryl monostearate added. Cake A3 was baked at 425°F.; B3, at 375°F.; C3, at 325°F.; and D3, at 300°F.

kind of fat used. Cakes baked at the higher temperature showed the most browning and least adherence of crust to the pan. At the lowest temperature there was most clinging of crust to the pan, and least browning, though the cakes remained in the oven for a comparatively long time.

Gumminess, smoothness of crust, and the presence of white dots were affected by oven temperature and by the kind of fat used and not by the presence of emulsifier.

Cakes baked at the higher temperatures had thin, tender crusts, while the crusts of those baked at low temperatures tended to be gummy and sticky. The crusts of vegetable shortening cakes baked at 300°F. were more gummy than those of butter cakes baked at this temperature.

Smoothness was the only crust characteristic that appeared to be affected by the presence of an emulsifier. The crusts of cakes without an emulsifier baked at 375° and 425°F. were blistered, whereas the crusts of cakes containing emulsifier were smooth and velvety in appearance. Blisters disappeared at the lower baking temperatures of 300° and 325°F.

White dots appeared in the crust of cakes baked at the lower temperatures. When these dots were observed at 36 magnifications they appeared to be sections of the crust that had been raised by gas or steam escaping from the cake. At these points the crust was dehydrated and had lost its gelatinous consistency. Spots occurred more frequently on cakes made with hydrogenated vegetable oil.

Palatability scores. Cakes were scored for the following characteristics: cell structure, compactness, tenderness, springiness, moistness, velvetiness, flavor, and overall acceptability. Average scores for palatability characteristics are shown in Table 4. The analysis of variance was used for cake scores. The method as given in Table 2 was followed, except that variance due to judges was included. The following significant differences were found.

The addition of emulsifier significantly improved cell structure, but no difference was found due to the incorporation of emulsifier as a fat or water dispersion, or the amounts of emulsifier used. The type of fat did not produce a significant difference in the scores for grain. Cell structure improved with each increase of baking temperature. The difference in grain between cakes made with and without emulsifier when baked at different temperatures may be seen in Figures 5 and 6.

Compactness and tenderness were scored lower for cakes without emulsifier, but there was no significant difference among cakes prepared with the 2 emulsifiers at 2 levels. Butter cakes were scored higher than cakes made with vegetable shortening. No significant difference was found among cakes baked at the 3 higher temperatures, but those baked at 300°F. received lower scores. The cakes baked at this temperature were compact, gummy or heavy when chewed, and excessively tender in the sense that the crumb broke easily rather than having a desirable elasticity accompanied by some breaking.

For springiness, moistness, velvetiness, and flavor, butter cakes were rated higher than cakes made with hydrogenated vegetable oil. This may be accounted for by the distinctive flavor of butter and the fact that butter dispersed better throughout the crumb. The presence of an emulsifier did not produce significant differences in cake scores for any of these characteristics. Each increase in baking temperature resulted in improved palatability.

The scores for overall acceptability indicated that butter was superior to hydrogenated vegetable oil when used as a cake shortening, and that cakes with emulsifier were preferred to those without, but cakes made with the 2 emulsifiers at the different levels were not distinguished from each other. Desirability increased with each increase in baking temperature.

Microscopic appearance of cake crumb. Microscopic observations revealed that a network of protein gave structure to the cake crumb. From the fibrous nature of the framework, it was concluded that flour gluten proteins were for the most part responsible for the structure.

The black-stained fat appeared for the most part as a coating on the pink-stained protein framework. Starch granules could be seen embedded in the filamentous protein structure and in the fat bordering the cells. Granules situated in the margin were partially covered with fat. When the sections were viewed between crossed polaroids, the presence of a few raw starch grains could be recognized.

It is probably during the last stages of baking, when expanding gas cells rupture the protein matrix, that many of the melted fat lakes run into the openings left by the gas bubbles. Each mobile fat lake might coat 1 or several bubbles, and penetrate the protein-starch matrix in varying degrees.

TABLE 4

Average scores for characteristics of cakes made with 2 fats, with and without glyceryl monostearate (GMS) emulsifier, and baked at different temperatures. Cakes compared with a reference standard having a score of 5.

	No emulsifier				GMS, fat-dispersed								GMS, water-dispersed							
					3%				6%				3%				6%			
Baking temperature, °F.	300	325	375	425	300	325	375	425	300	325	375	425	300	325	375	425	300	325	375	425
Butter																				
Characteristics																				
Thickness of cell walls	1.4	2.9	3.9	4.1	2.3	2.8	4.9	7.3	2.4	3.3	6.7	7.5	1.8	3.5	5.7	6.5	1.8	3.2	5.3	5.7
Cell distribution	1.6	3.1	4.4	3.9	2.7	3.3	4.9	6.2	2.5	3.5	6.3	7.1	2.3	4.3	5.5	6.1	2.3	3.5	5.3	5.1
Cell size	1.8	3.0	4.2	3.7	2.7	3.1	5.3	7.0	2.4	3.3	6.5	7.3	2.3	3.7	5.4	6.3	2.2	3.6	5.2	5.4
Springiness	2.4	4.3	4.3	3.8	2.1	3.6	4.7	6.1	2.0	3.2	5.0	6.3	2.1	3.4	4.7	5.0	1.6	4.0	5.2	3.9
Compactness	3.7	5.9	3.6	3.1	3.2	5.7	4.6	6.0	4.0	5.7	6.3	6.6	3.7	5.8	5.3	5.2	3.2	5.7	5.4	5.3
Tenderness	3.3	4.7	4.1	3.8	3.6	5.2	5.0	6.8	3.4	5.0	6.2	6.9	3.2	5.3	5.3	5.2	3.2	5.3	5.9	5.2
Moistness	2.4	3.7	4.7	5.3	2.1	3.3	5.0	5.7	2.3	3.6	4.3	5.0	1.8	3.6	4.8	4.8	1.8	3.4	4.4	4.7
Velvetiness	1.9	3.3	4.4	5.2	1.8	3.3	5.3	6.8	2.2	3.6	5.7	7.0	1.7	3.7	4.8	5.1	1.9	3.3	4.9	5.4
Flavor	4.3	4.0	4.8	4.4	4.0	4.6	4.9	5.8	3.8	4.4	5.0	5.3	3.3	4.2	4.7	5.0	3.3	4.2	4.8	4.6
Overall desirability	2.2	3.6	3.9	3.4	2.3	3.7	4.7	6.9	2.8	3.9	6.3	7.3	2.1	3.8	5.0	5.7	2.1	3.8	5.2	5.6
Vegetable shortening																				
Thickness of cell walls	1.9	2.7	3.8	4.6	1.6	3.7	4.7	6.1	1.8	3.7	5.3	6.6	2.1	3.2	4.7	6.0	2.2	3.6	4.3	6.5
Cell distribution	2.3	3.0	4.6	4.4	2.1	4.4	4.7	6.0	2.8	4.2	5.8	6.9	3.0	4.2	5.6	5.8	2.0	3.7	4.3	6.2
Cell size	2.3	3.2	4.8	5.4	2.2	4.0	4.9	6.0	2.3	4.2	5.8	7.2	2.8	3.8	5.3	6.1	2.2	3.9	4.3	6.2
Springiness	2.0	3.4	4.4	3.9	1.3	3.1	4.2	4.7	1.3	2.6	4.1	5.2	2.1	3.7	4.2	5.2	1.4	3.3	4.1	5.3
Compactness	2.8	3.8	3.7	3.8	2.6	5.1	5.0	4.0	2.7	4.9	4.9	5.9	3.4	5.9	5.0	5.8	2.3	5.6	4.7	5.6
Tenderness	3.4	4.6	3.8	3.7	2.9	4.7	5.3	4.9	2.3	3.8	5.1	6.0	3.4	5.2	5.1	5.3	2.9	4.3	5.2	6.3
Moistness	2.6	3.6	4.1	4.4	1.2	3.5	4.4	4.4	1.7	3.0	4.0	4.3	1.7	3.5	4.3	4.6	1.3	3.1	4.2	4.1
Velvetiness	2.2	3.2	4.0	4.6	1.2	3.3	4.4	5.0	1.3	3.2	4.2	5.1	1.6	3.1	4.4	4.8	1.4	3.2	4.4	5.1
Flavor	3.1	3.2	3.4	3.8	1.8	3.6	4.0	4.0	2.0	2.8	3.9	4.0	2.7	3.6	4.2	4.0	2.0	3.7	4.0	3.9
Overall desirability	2.3	3.1	3.2	3.9	1.3	3.4	4.4	4.4	1.7	3.1	4.1	5.6	2.0	3.2	4.4	5.1	1.4	3.3	3.9	5.0

Effect of fat and emulsifier. The distribution of butter appeared different from that of the hydrogenated vegetable fat. The butter covered the protein walls in a thin film which penetrated the cell wall structure following the gluten fibers. The hydrogenated vegetable oil on the other hand gathered in heavy beads along the protein walls. These beads could be differentiated, edging the protein structure in a discontinuous row. Although butter clumps could be noticed at the points of contact between cell walls, it was not typical of this fat to gather in clumps or beads. In sections of cake containing an emulsifier more fat appeared to be spread throughout the protein structure than when no emulsifier was present.

Effect of baking temperature. Microscopic observations of cake sections revealed thin cells walls and small air cells in cakes baked at high temperatures, as contrasted with thick cell walls and large air cells in cakes baked at low temperatures.

CONCLUSIONS

When glyceryl monostearate was added to a cake formula, the fat together with the air bubbles became more highly dispersed. Improved fat and air dispersal was undoubtedly brought about by the emulsifier concentrating at the fat-liquid and air-liquid interfaces. This stabilized the finely dispersed fat and air bubbles.

The stabilizing effect of the emulsifier on fat and air distribution was most effective when proteins were coagulated early in the baking process. This took place when cakes were baked at high temperatures. Cakes which contained emulsifier and were baked at high temperatures had better volume and received higher palatability scores than those which were baked at low temperatures. Cakes made with butter plus emulsifier had larger volume and received higher scores for most palatability characteristics than corresponding cakes made with hydrogenated vegetable shortening.

No difference in cake scores was found due to the percentage of emulsifier used (3 or 6%) or to its dispersal in fat or water.

LITERATURE CITED

1. Bailey, A. E. *Industrial Oil and Fat Products.* 1945, Interscience Publications Inc., New York.
2. Barmore, M. A. The influence of various factors including altitude, in the production of angel food cake. Colorado Agr. Expt. Sta., *Tech. Bull.*, **15** (1936).
3. Carlin, G. T. A microscopic study of the behavior of fats in cake batters. *Cereal Chem.*, **21**, 189 (1944).
4. Daum, Ruth, Halliday, Evelyn G., and Hinman, W. F. The effect of glyceryl monostearate on the baking properties of cakes. *Oil and Soap*, **19**, 39 (1942).
5. Johnston, N. F., and Favor, H. H. New emulsifiers for foods. *Food Inds.*, **19**, 1196 (1947).
6. Jooste, Martha E., and Mackey, Andrea Overman. Staining methods for studying ingredient distribution in baked cakes. *Stain Technol.*, **27**, 17 (1952).
7. Lowe, Belle. *Experimental Cookery.* 3d. Ed., 1943, John Wiley and Sons, New York.
8. Mackey, Andrea Overman. Cake formula and method of mixing for cakes made with different types of fat. Unpublished data. Department of Home Economics, Agricultural Experiment Station, Oregon State College.
9. Weiser, H. B. *Colloid Chemistry.* 2d Ed., 1949, John Wiley and Sons, New York.

SOME ASPECTS OF MICROBIOLOGY APPLIED TO COMMERCIAL APPLE JUICE PRODUCTION.

PART IV. DEVELOPMENT CHARACTERISTICS AND VIABILITY OF SPOILAGE ORGANISMS IN APPLE JUICE

C. R. MARSHALL AND V. T. WALKLEY
Seager, Evans and Co., Ltd., Deptford Bridge, London, England

(Manuscript received July 25, 1951)

All of the microorganisms found associated with apples and their juice that we have described in a previous paper *(1)*, can, if allowed to develop, promote varying degrees of spoilage in the juice. Under normal factory conditions their growth is retarded in juice stored in bulk by means of saturating the juice with carbon dioxide under pressure and maintaining the temperature of the juice as low as possible without freezing. Prior to bottling, the juice is given a polishing filtration which effects a considerable reduction in the number of these organisms, and we shall demonstrate in a subsequent paper that those organisms which survive are later destroyed by pasteurization of the juice in bottle. Exceptions can occur, however, for we have observed on occasions that a few odd bottles from a batch that has been pasteurized may contain a small number of viable mold spores which are capable of development if plated out on suitable media. It is indeed fortunate that in such cases the presence of a very small concentration of carbon dioxide is sufficient to render these organisms incapable of development in bottled juice, as will be shown in this paper.

We have studied the actual spoilage effects that these organisms can produce in apple juice and have recorded below the characteristic development of each species and its appearance in the bottled juice. This data may be utilized as an additional means of rapid visual identification of a contaminating organism, should bottles of infected apple juice be encountered during normal factory conditions.

It must also be borne in mind that accidents can arise during bulk storage of the juice, such as failure of the refrigeration plant with perhaps an accompanying increase in temperature of the juice before the breakdown can be repaired, or loss of carbon dioxide through faults developing in the tank fittings. Since one storage tank may hold several thousand gallons of juice containing a miscellaneous assortment of microorganisms, it is important to know to what extent the character of the juice may be affected during the period of the breakdown should some of these organisms become active while the retarding influences of gas pressure and low temperature are temporarily withheld or reduced.

Accordingly, a pure culture of each of the organisms that we have isolated and described in a previous paper *(1)* was inoculated into bottles of sterile apple juice, and its development and appearance in the bottled juice accurately recorded. Our data and records of each organism are too lengthy and detailed to be described fully in this paper. Consequently,

we shall confine our observations to a description of the technique and to the more salient points only concerning the development characteristics and spoilage effects of each group of organisms that has been examined.

METHOD

Each organism studied has been cultured on apple juice agar at 25°C. and before use was examined to ascertain that it still retained all the characteristics of the original isolate obtained from the plant.

The juice for inoculation was filled into 7-ounce flint glass mineral water bottles. The crown cork closures were specially prepared before use, the cork wads being removed and replaced by close fitting rubber discs of similar thickness. A hole, 1/32 inch in diameter, was drilled in the center of each crown before inserting the rubber discs. After filling with juice and crowning, all bottles were pasteurized to render juice, crown, and bottle sterile. A few bottles from each batch were set aside and the sterility of the juice confirmed by withdrawing samples and plating out on nutrient apple juice agar.

Apple juice manufactured by the Boehi process, which is in general use in Great Britain, invariably contains from 0.5 to 1.0 volumes of carbon dioxide when bottled. This is the residual content of gas remaining after the juice has been withdrawn from the storage tanks prior to final polishing filtration before bottling, and after the excess gas with which the juice has been impregnated throughout storage has been allowed to "blow off." We desired to examine whether this small residual content of carbon dioxide would have any repressive action on the growth of microorganisms in the bottle. Consequently, we adjusted the carbon dioxide contents of all the bottled juices to three different levels at S. T. P., namely, 0.75, 0.5, and 0.0 volumes.

After all bottles had been filled in the manner described above and their contents pasteurized, each bottle was inoculated aseptically with a pure culture of the particular microorganism isolated. In the case of the molds, only spore inoculations were used. All cultures had been grown on apple juice at 25°C.(77°F.). Suitably diluted suspensions in sterile water were prepared and inoculated into the bottles by means of a hypodermic syringe. The needle of the latter was inserted through the hole in the crown cork and used to pierce the rubber liner. In this manner, exposure of the juice in the bottle to external contamination was avoided, the rubber liner was self-sealing, and there was no loss of gas from the juice in those samples which still retained some degree of carbonation.

Then 6 bottles were inoculated with each organism, giving 2 pairs at each level of carbonation. One bottle of each pair was incubated at 25°C.(77°F.) and the other bottle allowed to remain at room temperature. All bottles were maintained under observation for periods up to 2 months and examined at regular intervals to record the growth characteristics of each organism in the bottled juice.

At the close of the storage period, samples of the juice were removed aspetically from each bottle by means of a hypodermic syringe, seeded on apple juice agar plates and incubated at 25°C.(77°F.). By this means a record was obtained of all organisms which, without necessarily having caused spoilage in bottle under the conditions of the test, still remained viable.

Since the development of organisms in the bottle at room temperature was identical with that which occurred at 25°C. only the latter are recorded in the following text.

RESULTS

Descriptions of the appearance and growth characteristics of each organism in bottled juice are too lengthy and detailed to be given fully in this paper. It is considered too, that development will be influenced to a certain extent by the composition of the juice and its pH value. Consequently, although the records obtained by us on blended juice that conforms to a fairly uniform standard from year to year in the factory where it is produced, have proved sufficiently consistent to act as a ready means of identification for routine work, such records would not necessarily be

strictly applicable for juice produced elsewhere. We have, therefore, presented a brief summary of the more important growth characteristics of each group of organisms. These should be generally consistent irrespective of minor variations in the composition of apple juice produced in different localities. Our main observations are as follows.

The Mucorales

During the period of observation (2 months), no species exhibited any growth whatsoever in the presence of 0.75 volumes of carbon dioxide. With a gas content of 0.25 volumes there was very slight evidence of restricted spore germination in *Mucor hiemalis* Wehmer and *Rhizopus nigricans* Ehrenberg. *Mucor racemosus* Fresenius developed a slight growth of submerged mycelia after 10 days forming a thin greyish-white deposit but no further development occurred subsequently. In the absence of carbon dioxide (completely aerobic juices) all species exhibited varying degrees of development within 7 to 10 days after inoculation. All species formed surface mycelia but with the exception of *M. racemosus* Fresenius and *R. nigricans* Ehrenberg, formation of submerged mycelia and fruiting structures was scanty. In general, after 2 months, apart from the surface mat submerged filaments settled as deposits at the bottom of the bottles and the juice cleared. *R. nigricans* Ehrenberg was the sole exception. Although none of the juices changed color, odor and flavor were affected to varying degrees.

Table 1 shows the viability of each organism after 2 months in the bottle when seeded on to apple juice agar.

TABLE 1

Viability of the Mucorales after two months in bottled apple juice

Organism	Volume CO_2 at S.T.P.		
	0.75	0.25	0.0
Mucor mucedo Linne	−	+[a]	+
Mucor pyriformis Fischer	−	+	+
Mucor racemosus Fresenius	+	+	+
Mucor hiemalis Wehmer	−	−	+
Rhizopus nigricans Ehrenberg	+	+	+

[a] The sign + indicates that the organism was viable.

The Aspergilli

None of the species investigated exhibited any growth in the presence of 0.75 volumes of carbon dioxide. Slight swelling of conidia in the presence of 0.25 volumes of carbon dioxide was shown only by *Aspergillus niger* (Strain B), *Aspergillus ustus*, *Aspergillus nidulans* Eidam, and *Aspergillus wentii*. No development was observed in any of the remaining species examined. There was no change in character, flavor, or bouquet of any of these juices.

Under aerobic conditions, each species commenced mycelium formation within 2 to 14 days. Surface mycelia eventually exhibited the color development characteristic of each individual species. Development of fruiting

structures was evidenced by all species, although varying in extent. Although in general the juices became hazy, most had clarified after 2 months. Changes in flavor varied from a pronounced objectionable taste and odor with a decrease in pH from 4.0 to 3.5 produced by *A. niger* (Strain A) to practically no change in the presence of *A. ustus*.

Table 2 shows the viability of each species after 2 months in the bottle.

TABLE 2

Viability of the Aspergilli after two months in bottled apple juice

Organism	Volume CO_2 at S. T. P.		
	0.75	0.25	0.0
Aspergillus niger Strain A	+[b]	+	+
Aspergillus niger Strain B	+	+	+
Aspergillus ustus	+	+	+
Aspergillus versicolor Vuillemin	−	+	+
Aspergillus nidulans Eidam	−	−	+
Aspergillus wentii	−	+	+
Aspergillus flavipes Bainier & Sartory	−	−	+
Aspergillus ochraceus Wilhelm	+	+	+
Aspergillus fumigatus	+	+	+
Aspergillus fischeri Wehmer	+	+	+

[b] The sign + indicates that the organism was viable.

The Penicillia

Ten species were examined and none showed any evidence of germination in the presence of either 0.75 or 0.25 volumes carbon dioxide.

Under aerobic conditions growth proceeded very rapidly with most species forming a heavy mycelial mat completely covering the surface of the juice, but in no case was any submerged growth detected. Each species developed a colony exhibiting the typical characteristics of the species when grown in pure culture. Germination and formation of a surface mycelium was latent in *Scopulariopsis* (*Scopulariopsis brevicaulis* Bainier), and this organism alone was characterized by sparse growth of aerial hyphae but with little development of conidiophores. The juice below the surface mycelia of all species remained perfectly clear during the period of observation with no color change except with *Penicillium viridicatum* Westling which caused the juice to darken. Most species caused some slight increase in acidity and also imparted varying degrees of mustiness to the flavor and odor. *Paecilomyces varioti* Bainier induced development of an off-flavor somewhat reminiscent of pineapple.

All the ten species examined remained viable after two months of storage in bottled apple juice under aerobic conditions and also in the presence of concentrations of 0.25 and 0.75 volumes of carbon dioxide. The organisms examined were *Penicillium expansum* (Link) Thom, *Penicillium cyclopium* Westling, *Penicillium crustosum* Thom, *Penicillium viridicatum* Westling, *Penicillium glabrum* (Wehmer) Westling, *Penicillium spinulosum* Thom, *Penicillium cyaneum* (Bainier and Sartory) Biourge, *Lanata-typica* group (species unidentified), *Scopulariopsis brevicaulis* Bainier and *Paecilomyces varioti* Bainier.

The Fungi Imperfecti

The species within this group exhibited more diversity in their development in bottled juice than those of any other group examined.

Fusarium under both aerobic and non-aerobic conditions showed no tendency to develop whatsoever, either at room temperature or at 25°C. (77°F.).

Germination of the spores of *Alternaria tenuis* commenced within 7 days under aerobic conditions but was slightly retarded in the presence of carbon dioxide and the quantity of mycelium produced was scanty. In the absence of carbon dioxide a rich surface growth of dark green conidia developed but after 3 weeks showed signs of degeneration. The juice acquired a musty flavor and odor and became slightly turbid.

Development of *Cladosporium herbarum* was very restricted in the presence of carbon dioxide but aerobically showed development within 7 days, imparting a pronounced objectionable flavor to the juice.

In the presence of carbon dioxide, *Botrytis cinerea* developed rudimentary mycelia followed by complete cessation of growth. A surface colony with characteristic fruiting structures developed in carbon dioxide free juices. Also, 0.75 volumes carbon dioxide was insufficient to check the growth of *Oospora lactis*, which developed rapidly in the absence of carbon dioxide and although showing preference for development of a white surface mycelium it also appeared as a white, slimy growth at the bottom of the bottle. Its growth was affected considerably by the pH of the juice.

In the presence of carbon dioxide, growth of *Oospora candida* was restricted to the formation of a few rudimentary cells at the bottom of the bottle. In absence of carbon dioxide, growth was rapid with early surface growth of cells and mycelium. Gas was formed from ooidium fragments which sank to the bottom of the bottle. There was loss of sugar in the juice accompanied by formation of alcohol. The juice appeared thin and acid, with slight off-flavor development and some change in color.

Only Fusarium, among the six species of this group that were examined, lost its viability after two months of storage in apple juice bottled under both aerobic and anaerobic conditions.

The Yeasts

Various yeasts isolated from apple juice, comprising cider yeasts, *Torulae* and *Rhodotorulae*, remained viable after several months in bottled juice. All the cider yeasts produced vigorous fermentation with formation of alcohol accompanied by a rise in pressure after several weeks up to 60 lbs. p.s.i.g. Although small initial gas contents of 0.25 and 0.75 volumes did not hold the yeasts in check, it was noted that some strains imparted pleasant cider characteristics to the juice, which was not always so in the juices that were originally carbon dioxide free.

The *Torulopsis* yeasts did not appear to have significant spoilage qualities and in juice bottled with initial carbon dioxide contents of 0.25 and 0.75 volumes, although the cells were still viable after 2 months, there was no significant increase in numbers and no deterioration in flavor of the juice. Under aerobic conditions, their growth was slow, exhibiting haze

with formation of cell sediments after 5 days. There was no gas formation, no surface growth, and no appreciable spoilage of the juice quality.

The *Rhodotorulae* examined, produced no gas in the presence of 0.25 and 0.75 volumes carbon dioxide, and showed only slight development up to 2 months in bottle. No surface growth was encountered and although some foreign "bouquet" was imparted to the juice, no other spoilage factor was detected.

The Bacteria

Development of *Acetobacter xylinum* Brown was completely checked in the presence of 0.75 volumes of carbon dioxide. In the presence of 0.25 volumes of carbon dioxide, a surface pellicle had formed by the 14th day, with formation of gelatinous threads in the juice. There was a slight decrease in sugar content of the latter but the flavor was unimpaired. Growth was more vigorous in the carbon dioxide free juice with the development of a pronounced off-flavor.

Growth of *Acetobacter oxydans* was very restricted in the presence of 0.25 and 0.75 volumes of carbon dioxide and the juice suffered no deterioration in flavor. Under aerobic conditions a slight haze developed throughout the juice within 3 days and after 7 days a thin surface film developed. After 2 months the juice lacked a little astringency but otherwise was of excellent flavor.

It was noted that 0.75 volumes carbon dioxide completely checked the growth of *Lactobacillus delbrueckii* (Leichmann) and in the absence of carbon dioxide growth was restricted to a slight white surface film. No off-flavor impaired the taste of the juice and no change in acidity was detected.

Both *Lactobacillus plantarum* (Orla Jensen) and *Lactobacillus leichmannii* (Bergey) showed only slight development in both carbonated and non-carbonated juice and neither organism had any deleterious effect on the flavor.

These organisms were only maintained under observation in bottled juice for 21 days before plating out and testing for viability. *A. xylinum* Brown that had been exposed to a concentration of 0.75 volumes of CO_2 at S.T.P. failed to survive, but at 0.25 volumes concentration and also in the absence of CO_2, growth developed when plated out. The four remaining species of bacteria were viable after storage in both still and carbonated juice.

SUMMARY

With the exception of the yeasts, the majority of the isolates examined were unable to develop in the presence of 0.75 volumes of carbon dioxide. Among the *Fungi Imperfecti*, however, *Botrytis cinerea* and *Oospora candida* were able to show restricted development, whilst *Oospora lactis* was only slightly affected by the same concentration of carbon dioxide. Among the bacteria, only *Acetobacter oxydans* showed some slight development.

It was found that 0.25 volumes of carbon dioxide completely arrested development of all species of *Penicillia* examined. Organisms within the other groups showed extreme variation in their ability to germinate in the presence of this amount of gas.

Under anaerobic conditions all organisms with the exception of *Fusarium* exhibited varying degrees of development. *Lactobacillus delbruckii, Lactobacillus plantarum* and *Lactobacillus leichmannii* showed but slight development, no doubt due to restricted oxygen supply, and exerted no adverse effect on the flavor of the juice.

All the *Penicillia* and the *Fungi Imperfecti* with the exception of *Fusarium,* still remained viable after both aerobic and anaerobic storage conditions. Species within the other groups of organisms exhibited differences in their ability to survive when plated out on apple juice agar.

It will be apparent, therefore, that provided a slightly carbonated juice is bottled, it is not essential to prolong the pasteurization beyond the point at which the yeasts and *Botrytis, Oospora candida* and *Oospora lactis* among the *Fungi Imperfecti,* and *Acetobacter oxydans* are destroyed. The presence of 0.75 volumes of carbon dioxide will completely inhibit the development of any other organisms that may still survive.

Acknowledgment

This paper is published by kind permission of the Directors of Messrs. Seager, Evans & Co., Ltd., to whom the authors desire to express their thanks.

LITERATURE CITED

1. Marshall, C. R., and Walkley, V. T. Some aspects of microbiology applied to commercial apple juice production. Part III. Isolation and identification of apple juice spoilage organisms. *Food Research,* **17**, 123 (1952).

SOME ASPECTS OF MICROBIOLOGY APPLIED TO COMMERCIAL APPLE JUICE PRODUCTION.

PART V. THERMAL DEATH RATES OF SPOILAGE ORGANISMS IN APPLE JUICE

C. R. MARSHALL AND V. T. WALKLEY
Seager, Evans and Co., Ltd., Deptford Bridge, London, England

(Manuscript received July 25, 1951)

More has probably been written on pasteurization than on any other aspect of apple juice production. Since current practice in Europe favors "holding" pasteurization, this method only has been studied in detail by the authors.

A brief resumé of the literature will reveal the difference of opinion that has existed concerning the most favorable temperature and holding time for pasteurization of bottled juice. Cruess and Irish *(2)* reported that apple juice could be sterilized at 150°F.(65.5°C.) for 30 minutes but carbonated juice was said to require a temperature of 170°F.(76.5°C.) for the same period. Poore *(7)* pasteurized in bottle at 172°F.(78°C.) and then cooled quickly with either water or a fan. Fabian and Marshall *(3)* found that the pasteurization time of apple juice was determined to a certain extent by the number and type of organisms present in the juice. The vegetative forms of yeast were killed at a temperature of 150°F. (65.5°C.) in 10 minutes. The most resistant mold spores were killed by pasteurization at a temeprature of 175°F.(79°C.) for 20 minutes and certain bacteria at 158°F.(70°C.) for 30 minutes or 176°F.(80°C.) for 15 minutes. They also declared that acidity was an important factor in determining the time and temperature to be used in pasteurization.

Cruess, Aref, and Irish *(1)* stated that the death temperature of yeast is 140-149°F.(60-65°C.). Mold spores, however, often withstand temperatures of 167-176°F.(75-80°C.). These mold spores require oxygen for germination and growth, and if air is well removed from the fruit juice by washing with a stream of carbon dioxide and is then carbonated to 15 p.s.i.g. pressure or more, the juice can be kept indefinitely by pasteurizing at 150°F.(65.5°C.) or less. They studied the time required to kill all the cells present in apple juice and found that at 134°F.(57°C.), all cells were killed in 10 minutes, but at 128°F.(53°C.), 120 minutes was required to secure effective pasteurization. They also found that nitrogen and carbon dioxide further reduced the temperature required for pasteurization but the effect was relatively slight. Juices heavily inoculated with yeast cells and heat resistant mold spores kept perfectly when pasteurized at 140°F.(60°C.), for 30 minutes if: a, carbonated before bottling; or b, if vacuumized and the vacuum released with nitrogen and the juice was charged with nitrogen during bottling.

Pederson and Tressler *(6)*, over a 20-minute heating period during which the temperature of apple juice was raised from 39-177°F.(4-80.5°C.),

took samples at minute intervals and carried out plate counts on the numbers of organisms surviving. The original juice had a count of 175,000 organisms per ml. After 14 minutes of heating in which the juice had attained a temperature of 154°F.(62°C.), all organisms were destroyed. They also showed that in presence of a preservative organisms are killed at a lower temperature. A concentration of 0.1% sodium benzoate or 20 p.p.m. sulphur dioxide may lower the temperature necessary for pasteurization as much as 10-15°F.(5.5-8.5°C.).

Tressler, Joslyn, and Marsh *(8)*, after reviewing the literature, made the following recommendations. Yeasts and most acid tolerant bacteria are destroyed by heating to 150°F.(65.5°C.) for a few minutes. In strongly acid juices the death temperature is 130-135°F.(54.5-57°C.). Some spore-forming bacteria require heating to 190°F.(88°C.). Mold spores require 175°F.(80°C.) for 6-10 minutes. For low temperature pasteurization of carbonated apple juice they recommend holding at 140°F.(60°C.) for 30 minutes or 130°F.(54.5°C.) for 2 hours.

Similar disagreement among different investigators has also been recorded for the pasteurization of beer. We ourselves hold similar views to Lund *(4)* who pointed out that this may be due to species of the same genus and strains of the same species reacting differently to temperature, also to the medium in which the organism used for the experiment is grown, the type of beer to be heated, the rate at which the temperature is reached, and the duration of the heating process.

We maintain that the satisfactory pasteurization of apple juice in bottle can only be achieved if large scale experiments are carried out in the pasteurization plant itself under actual processing conditions. The rate of heating must be carefully controlled and standardized for each size of bottle used. A holding temperature must be selected that will secure adequate pasteurization of the juice within a reasonably short time without causing any impairment of flavor. The time required to maintain the juice at the holding temperature must be determined by ascertaining the thermal death point of every species of microorganism known to be generally encountered in the juice processed at the factory. The data obtained under such conditions will be specific for the plant and for juice of the composition produced in that plant, and will not necessarily be generally applicable elsewhere.

The results given in this paper are a selection from many pasteurization experiments carried out by the authors under factory conditions. They are presented to illustrate that complete control of apple juice pasteurization in bottle may be achieved by application of the fundamentals of microbiology.

METHODS

Before describing the technique that has been employed in this investigation we feel it would be wise to discuss a little more fully our reasons for selecting the technique adopted.

In general, other investigators have either selected a pre-fixed holding temperature and time, and then ascertained whether juice bottled under factory conditions was sterile after pasteurization, or else known quanti-

ties of a mixed culture of microorganisms have been inoculated into fixed volumes of the juice and the latter slowly heated up to a fairly high temperature. Samples have been withdrawn at intervals during the heating period, seeded on to nutrient agar plates and the temperature at which no organisms survived was recorded.

We prefer to select a pre-determined holding temperature and examine the time that the juice must be held at that temperature for all the microorganisms to be destroyed. We do consider it important, however, to provide against the contingency that the samples of juice used for the test may not contain a representative collection of all the microorganisms generally to be encountered in the factory. Hence, we use pasteurized juice for our tests and inoculate with single cultures of each organism that we have encountered in the course of routine microbiological analysis of our juice, and determine the individual death times of each organism. Thus it is possible to establish conditions that will secure adequate pasteurization for each batch of juice irrespective of the composition of its micro-flora.

Containers. For the experiments described in this paper, the juice was filled into 7-ounce flint glass bottles and closed with crown corks. The cork liners of the latter were replaced with rubber discs of similar thickness, and a hole drilled in the center of each crown of sufficient diameter to enable the needle of a hypodermic syringe to be inserted. Each bottle of juice was pasteurized before use and the sterility of the contents of each batch was confirmed by selecting bottles at random, withdrawing samples and seeding on to apple juice agar plates.

Organisms used for tests. First 14-day cultures on apple juice agar of each organism were prepared, a suspension made in sterile water, and inoculated into each bottle of sterile juice by means of a hypodermic syringe. After withdrawing the needle, the rubber liners in the crowns enabled each bottle to become self-sealing and gas-tight.

Immediately after inoculation and thorough shaking to disperse the inoculum throughout the juice, samples were withdrawn by means of another sterile hypodermic syringe and seeded on to nutrient apple juice agar plates to enable a count to be made of the number of organisms inoculated per ml. of juice.

Pasteurization. The pasteurizer used was a full size commercial plant of the total immersion type in which the water having attained the desired pasteurization temperature, was held at that temperature for the requisite time by thermostatic control.

The bottles were placed horizontally in the pasteurizer and the rate of heating carefully standardized and rigidly controlled so that the rate of heat transfer was identical for each series of experiments. With juice at an initial temperature of 60°F. (15.5°C.), a uniform temperature of 145°F.(63°C.) throughout the bottle was attained after 23 minutes.

At 5-minute intervals from the commencement of heating, 1-ml. samples of juice were withdrawn from each bottle using a sterile hypodermic syringe, and after making known dilutions in sterile water, transferred to nutrient apple juice agar plates and incubated at 77°F.(25°C.). After 48 hours the colonies on each plate were counted and the equivalent number of viable organisms in the original juice calculated. All plates were then returned to the incubator for another 5 days and a second enumeration of the colonies made. A 48-hour incubation period was usually found to be sufficient for all viable organisms to develop.

RESULTS

The juice used possessed the following composition: specific gravity, 1.050; malic acid, 0.93%; pH, 3.2; total reducing sugars, 10.5%; tannin, 0.16%; and carbonation, 0.75 volumes at standard temperature and pressure.

A 182-ml. quantity of juice was filled into each bottle allowing a headspace of 14 ml. at 60°F.(15.5°C.).

The number of organisms selected for each inoculation was certainly not less than the number usually encountered in apple juice at the bottling stage, and invariably was in much greater excess. It might be anticipated that increasing the size of the inoculation might have some slight influence on the thermal death-point. Preliminary experiments with *Aspergillus niger* showed that within the range of 200-900 organisms per ml., which is well above the range of that normally encountered in juice at the bottling stage, no difference in the mortality point was discernible. The yeasts, likewise, were not affected within the range normally encountered. The only exception proved to be the acetic acid bacteria where between the extreme range of 100-10,000 organisms per ml. a slightly longer time was required to destroy the organisms at the higher concentrations.

In Great Britain, the customary holding temperature for pasteurization of apple juice in bottle varies from 145-152°F.(63-66.5°C.). We selected 145°F.(63°C.) as the holding temperature for the current investigation and in the following tables the times required from commencement of heating apple juice at an initial temperature of 60°F.(15.5°C.) to destroy each species of microorganism selected are recorded.

TABLE 1

Thermal death-points of the Mucorales

Organism	Number of viable organisms per ml. of juice						
	Minutes						
	0	5	10	15	20	25	30
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)	63°C. (145°F.)
Mucor mucedo Linne	910	880	820	40	1	0	0
Mucor pyriformis Fischer	780	800	770	90	10	0	0
Mucor racemosus Fresenius	1300	1100	1400	30	3	0	0
Mucor hiemalis Wehmer	540	520	520	210	20	5	0
Rhizopus nigricans Ehrenberg	390	320	310	15	6	0	0

TABLE 2

Thermal death-points of the Aspergilli

Organism	Number of viable organisms per ml. of juice					
	Minutes					
	0	5	10	15	20	25
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)
Aspergillus niger Strain A	930	960	920	40	18	0
Aspergillus ustus	1300	1400	1100	40	0	0
Aspergillus nidulans Eidam	1400	1200	1400	400	6	0
Aspergillus wentii	1700	1500	1300	180	3	0
Aspergillus flavipes Bainier & Sartory	2000	1800	2200	600	4	0
Aspergillus ochraceus Wilhelm	80	100	100	2	0	0
Aspergillus fumigatus	370	390	370	150	75	0

TABLE 3

Thermal death-points of the Penicillia

Organism	Number of viable organisms per ml. of juice							
	Minutes							
	0	5	10	15	20	23	25	30
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)	63°C. (145°F.)	63°C. (145°F.)
Penicillium expansum (Link) Thom	960	980	890	40	3	0	0	0
Penicillium cyclopium Westling	420	480	200	18	0	0	0	0
Penicillium crustosum Thom	640	660	180	40	12	3	2	0
Penicillium viridicatum Westling	300	320	250	240	60	35	18	0
Penicillium frequetans Westling	780	760	800	510	40	25	0	0
Penicillium spinulosum Thom	130	150	130	115	90	....	3	0
Paecilomyces varioti Bainier	520	480	350	40	14	0	0	0
Lanata-typica group (Species unidentified)	830	820	780	180	40	14	0	0

In Table 4 it was impossible to make an accurate count of *Alternaria tenuis*. The organism is multicellular within a capsule, and each cell can become the progenitor of another organism. The capsule cannot be disrupted by agitation of the inoculum. Approximations are also given for the counts of both species of *Oospora* since it was found difficult to prepare plates on which each colony appeared as a distinct and separate entity.

TABLE 4

Thermal death-points of the Fungi Imperfecti

Organism	Number of viable organisms per ml. of juice						
	Minutes						
	0	5	10	15	20	25	30
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)	63°C. (145°F.)
Cladosporium herbarum	830	880	840	520	140	4	0
Botrytis cinerea	1600	1300	1400	1100	200	6	0
Monilia sitophilia	580	420	560	380	87	0	0
Alternaria tenuis	<500	<500	<500	<200	<20	0	0
Oospora lactis	>200	>200	>200	<100	2	0	0
Oospora candida	> 80	> 80	> 80	< 30	<10	1	0

TABLE 5

Thermal death-points of the yeasts

Organism	Number of viable organisms per ml. of juice					
	Minutes					
	0	5	10	15	20	23
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)
Cider yeast 1	35200	33000	27200	20	3	0
Cider yeast 2	62000	61800	3170	30	4	0
Cider yeast 3	21500	20500	2120	5	0	0
Cider yeast 4	13400	13000	980	10	0	0
Cider yeast 5	12000	12500	1800	20	5	0
Torulopsis	920	950	380	10	0	0
Rhodotorula T36	1420	1300	1200	130	1	0
Rhodotorula T39	3720	4000	240	10	0	0

TABLE 6

Thermal death-points of the bacteria

Organism	Number of viable organisms per ml. of juice				
	Minutes				
	0	5	10	15	20
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	56.5°C. (134°F.)	62°C. (144°F.)
Lactobacillus delbrueckii (Leichmann)	9100	11300	10600	100	0
Lactobacillus plantarum (Orla Jensen)	17130	15800	17600	380	0
Lactobacillus leichmannii (Bergey)	11600	12100	11800	8600	0
Acetobacter xylinum Brown	120	110	87	0	0
Acetobacter xylinum Brown	9400	9500	7300	15	0
Acetobacter oxydans	140	108	86	0	0
Acetobacter oxydans	8500	8520	3500	40	0

Comparing the organisms as groups, the bacteria succumbed more readily to heat treatment, their thermal death-points being below 144°F. (62°C.). With counts not exceeding 150 per ml. the *Acetic* bacteria were destroyed at 134°F.(56.5°C.). The yeasts required a maximum heating period of 23 minutes at which point the holding temperature of 145°F. (63°C.) was just attained.

The thermal death-points of the *Aspergilli* were very close to those of the yeasts. The *Penicillia* were of interest since wide diversity was shown between the species, for their mortality times varied from 20 minutes for *Penicillium cyclopium* to 30 minutes for *Penicillium crustosum, Penicillium viridicatum,* and *Penicillium spinulosum.* Among the *Mucorales, Mucor hiemalis* was the most heat resistant. Of the *Fungi Imperfecti, Alternaria tenuis* and *Oospora lactis* were a little more sensitive to treatment than the other members of the group.

One can conclude from these results that a total heating time of 30 minutes would be adequate to secure efficient pasteurization of juice in 7-ounce bottles under the conditions of the investigation. This includes a 23-minute warming up period and a holding period of 7 minutes at 145°F.(63°C.) which is much less than that generally regarded as being sufficient.

We concluded our investigation by carrying out some pasteurization tests on juice that had been held in Boehi pressure tanks for several months. The 7-ounce bottles were filled with juice and pasteurized in the usual manner. As an additioanl precaution, the holding period was extended a further 5 minutes, making a total heating time of 35 minutes. Samples were taken at intervals and counts made of the yeasts and molds.

The results of many experiments showed that the molds were not always completely destroyed in 35 minutes. At the end of this time the numbers varied from 0-2 per ml.

Results of 2 series of experiments are given in Table 7. Among 103 bottles examined after 35 minutes of pasteurization, 27 bottles contained 1 viable mold per ml.; 16 bottles contained 2 viable molds per ml.; and 60 bottles contained no viable mold per ml.

TABLE 7

Thermal death-points of heterogeneous microflora present in tank-stored juice as determined in 2 tests.

Organism	Number of viable organisms per ml. of juice								
	Minutes								
	0	8	10	12	15	20	25	30	35
	15.5°C. (60°F.)	32°C. (90°F.)	35.5°C. (96°F.)	42°C. (108°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)	63°C. (145°F.)	63°C. (145°F.)
Yeasts	16400	16000	3610	1500	8	0	0	0	0
Molds	24	21	20	20	14	2	0	0	0
Organism	Minutes								
	0	5	10	12	15	20	25	30	35
	15.5°C. (60°F.)	21.5°C. (71°F.)	35.5°C. (96°F.)	42°C (108°F.)	56.5°C. (134°F.)	62°C. (144°F.)	63°C. (145°F.)	63°C. (145°F.)	63°C. (145°F.)
Yeasts	16000	15300	1550	....	10	1	0	0	0
Molds	22	20	22	....	16	3	1	1	1

The majority of the spores resistant to pasteurization proved to be *Penicillia*. There were 6 strains of unidentified species isolated and cultured on apple juice agar. Then 7-day cultures of these strains were subsequently inoculated into sterile apple juice in 7-ounce bottles and submitted to pasteurization for 35 minutes. Fairly heavy inoculations were used, the number of spores for seeding the sterile apple juice varied from 4 to 530 times the numbers normally encountered in juice at the bottling stage.

After pasteurization, samples were withdrawn and seeded on to apple juice agar plates. Subsequent incubation failed to produce any growth, indicating that these organisms had now lost the heat resistance of the original spores. We can only assume that under the adverse conditions prevailing in the juice storage tanks, the spores of some species become toughened which would account for their increased resistance to heat treatment.

DISCUSSION AND SUMMARY

Under the conditions used for these tests, the thermal death-times of each microorganism, when examined individually, was less than 30 minutes from the commencement of heating. The viability of some of the organ-

isms might be expected to exceed this period if the juice were processed in larger bottles, and also if its composition differs from that used in the experiments described.

We have also demonstrated that in tank-stored juice, certain species of *Penicillia* may develop some resistance to heat treatment. It is not essential, however, to prolong the pasteurization period to inhibit these organisms completely, for we have established in an earlier investigation *(5)* that their development in bottled juice is completely restricted in the presence of 0.75 volumes of carbon dioxide. These members of the *Fungi Imperfecti (Botrytis cinerea, Oospora lactis,* and *Oospora candida)* which can develop under anaerobic conditions are fortunately inactivated after a 25-minute heating period. Incidentally, *Botrytis cinerea* proved to be the most heat resistant of all the *Fungi Imperfecti* examined.

Acknowledgment

The authors wish to express their thanks to the Directors of Messrs. Seager, Evans and Co., Ltd., for granting permission to publish these results.

LITERATURE CITED

1. Cruess, W. V., Aref, H., and Irish, J. H. Pasteurization investigations. *Fruit Products J.*, **12**, 358, 377 (1933).
2. Cruess, W. V., and Irish, J. H. Fruit beverage investigations. Calif. Agr. Expt. Sta., *Bull.*, **359**, 526 (1923).
3. Fabian, F. W., and Marshall, R. E. How to make, clarify, and preserve cider. Mich. State Expt. Sta., *Bull.*, **98** (1935).
4. Lund, A. Thermal death points of microorganisms in beer. *J. Inst. Brewing*, **52**, 307 (1946).
5. Marshall, C. R., and Walkley, V. T. Some aspects of microbiology applied to commercial apple juice production. IV. Development characteristics and viability of spoilage organisms in apple juice. *Food Research*, **17**, 197 (1952).
6. Pederson, C. S., and Tressler, D. K. Flash pasteurization of apple juice. *Ind. Eng. Chem.*, **30**, 954 (1938).
7. Poore, H. D. The production of apple juices, concentrates, and syrups. *Fruit Products J.*, **14**, 170 (1935).
8. Tressler, D. K., Joslyn, M. A., and Marsh, G. L. *Fruit and Vegetable Juices.* 1st Ed., 1939, Avi Publishing Co. Inc., New York.

A COMPARISON OF TWO METHODS FOR DETERMINING THE COLLAGEN CONTENT OF COOKED MEAT

RUTH M. GRISWOLD[a] AND FLORENCE LEFFLER
Department of Home Economics, University of Chicago, Chicago, Illinois

(Manuscript received July 30, 1951)

During a study of the effect of various methods of cooking beef round on the collagen content of the meat, the method of Hartley and Hall *(4)* was used for the first 31 cooked samples. Since collagen is hydrolyzed to gelatin during cooking, losses in collagen were expected for all the cooking methods. Therefore it was surprising to find that 21 of these cooked samples appeared to contain more collagen nitrogen, expressed as percentage of total nitrogen, than did the matched raw samples when analyzed by this method. Similarly, when the total amounts of collagen in the slice of round before and after cooking were compared, 19 of these 31 samples appeared to have gained collagen.

EXPERIMENTAL METHODS

To study the analytical method, qualitative tests (Millon, Hopkins-Cole, xanthoproteic) were made on the filtrate obtained after autoclaving the samples. Since the Hartley-Hall method assumes that gelatin is the only source of nitrogen in this filtrate, and since gelatin contains no tryptophane and only a trace of tyrosine *(2)*, negative tests for these two amino acids were expected. However, qualitative tests of the filtrates from four raw samples indicated some contamination, while tests of six cooked samples indicated considerable contamination with tyrosine and tryptophane. Parallel tests of a solution of commercial gelatin calculated to contain twice as much nitrogen as the filtrates were negative. These tests appeared to indicate that the apparent gains of collagen found on cooking some of the samples were due to decomposition of proteins other than collagen during autoclaving. This decomposition of non-collagen proteins was evidently greater in the cooked than in the raw samples.

Because of these findings, the method of Lowry, Gilligan, and Katersky *(5)* seemed a good choice for studying the effect of cooking on the collagen content of meat. In this method, protein other than collagen and elastin is removed by solution in 0.1 *N*. sodium hydroxide before autoclaving. When the method was followed as published, the last washing with sodium hydroxide gave a positive biuret test, and qualitative tests of the filtrate obtained after autoclaving were faintly positive for tyrosine and tryptophane. If washing with sodium hydroxide was continued until a negative biuret test was obtained on the last filtrate, indicating the removal of all protein other than connective tissue, tests on the filtrate obtained after autoclaving were negative for tyrosine and tryptophane. However when washing with sodium hydroxide was done according to the original directions, followed by washing with water to a negative biuret test, qualitative tests for tyrosine and tryptophane on the filtrate obtained after autoclaving were again positive. Evidently, non-collagen protein decomposed during autoclaving when the washing with sodium hydroxide was incomplete. Because of this fact, the method was modified to include washing with sodium hydroxide until a negative biuret test was obtained on the filtrates. The volume of 0.1 *N*. sodium hydroxide used for each washing was 20 times the weight of the meat. As would be expected, lower collagen figures were obtained after the extra washing than with the original method.

[a] Present address: Department of Home Economics, Indiana University, Bloomington, Indiana.

Several other slight modifications in the method were made. To minimize sampling errors, meat which had been ground three times before freezing was ground again while still frozen. To avoid fluctuations of temperature, samples were held at 26-27°C. instead of at room temperature while they were soaked overnight in sodium hydroxide. Since fat was not trimmed from the samples used, a fatty layer which occluded some of the fibers of connective tissue formed during the alkali washings. To avoid trouble from this source, the fatty layer was pushed aside before removing the supernatant liquid after the first addition of sodium hydroxide. After the second addition of alkali, 10 ml. ether was added, making it possible to remove the fat without occluded connective tissue.

Autoclaving was carried out at 20-25 p.s.i.g. for 6 hours, as suggested by Lowry, Gilligan, and Katersky *(5)*. Lower results were obtained when the material was autoclaved for 3 instead of 6 hours.

To test the possibility that the drying of the protein residue before autoclaving required by the method of Lowry, Gilligan, and Katersky might change its solubility, the nitrogen content of the filtrate removed after autoclaving was determined when the residue was dried according to directions, and also when drying was omitted. Approximately the same nitrogen content was found by these two methods, indicating that drying had little, if any, effect on the solubility or hydrolysis of the collagen.

RESULTS

The methods of Hartley and Hall and of Lowry, Gilligan, and Katersky, using the modifications described, were tested on purified collagen[b] from the corium of steer hide. This material, as received, contained 10.5% moisture, 2.7% fat, and 16.1% total nitrogen. As indicated in Table 1, its collagen content, calculated on the fat-free dry weight basis, was 99.0% by the Hartley-Hall and 94.2% by the Lowry method. The difference between the results by the two collagen methods may be more apparent than real, since it is necessary to use a factor for converting collagen nitrogen to collagen in the Hartley-Hall method. The factor 5.38 was used, which corresponds to a nitrogen content of 18.6%. This is the value found, on the fat-free, dry weight basis, for the collagen preparation used in this paper, and by others *(1, 3)*. It is possible, of course, that the collagen preparation contained some non-collagen protein which was determined by the Hartley-Hall, but not by the Lowry method.

Collagen data obtained by the two methods for several groups of matched raw and cooked samples from the semitendinosus muscle of beef round are also presented in Table 1. It is evident that results by the Hartley-Hall method are higher for the raw samples than those by the Lowry method. The results of the qualitative tests already reported suggest that the Hartley-Hall data are high due to the inclusion of some non-gelatin nitrogen with the gelatin nitrogen determined in this method. The analyses of steer-hide collagen indicate that when contaminating proteins are absent, or present only as traces, results by the two methods check within 5%. These analyses of purified collagen also show that if the Lowry method causes any losses of collagen, they are not large enough to explain the discrepancy between the assays on meat by the two methods.

---

[b]The authors are grateful to Dr. Fred O'Flaherty of the University of Cincinnati for furnishing this collagen. It was prepared in his laboratories according to the method of Buechler, as reported by Jacobs, J. L., in *J. Am. Leather Chemists' Assoc.*, **44**, 722 (1949).

TABLE 1

Collagen content of steer-hide collagen and of raw and cooked beef round analyzed by the methods of Hartley and Hall, and of Lowry, Gilligan, and Katersky

Sample	Moisture	Method of Hartley and Hall		Method of Lowry et al.
		Collagen N as % total N	Collagen [c]	Collagen
	%	%	%	%
Steer-hide collagen, dry, fat-free	00.0	99.0	99.0	94.2
Beef round, group [e] A				
Raw	71.8	12.1	2.32	0.87
Cooked, method 1	56.9	14.1 [d]	3.96	0.61
Cooked, method 2	64.4	15.2 [d]	3.24	0.79
Cooked, method 3	56.9	11.0	3.20	0.33
Beef round, group [e] B				
Raw	71.5	12.3	2.38	1.11
Cooked, method 4	56.7	14.1 [d]	3.54	0.93
Cooked, method 5	59.7	11.9	3.32	0.70
Cooked, method 6	58.1	13.1 [d]	3.98	0.56
Cooked, method 7	58.4	13.6 [d]	4.03	0.58
Beef round, group [e] C				
Raw	68.8	14.0	2.55	1.17
Cooked, method 8	53.5	13.0	3.34	0.61
Cooked, method 9	53.9	12.9	3.44	0.40
Cooked, method 10	57.4	16.1 [d]	3.66	0.48

[c] The % collagen equals collagen N as % total N × (% total N/100) × 5.38.

[d] Showed an apparent gain in collagen nitrogen during cooking when the total amounts in the matched slices of round before and after cooking were compared by calculating as follows: Weight of collagen N in raw meat equals % total N in raw × collagen N as % total N in raw × weight raw sample used for cooking. Weight of collagen N in cooked meat equals % total N in cooked × collagen N as % total N in cooked × weight cooked meat.

[e] Slices for each group of samples were close together in the round, so that the raw sample could be compared with samples within the same group which were cooked by different methods. The details of these cooking methods will be described in a future publication.

The table indicates that more than half of the meat samples showed on cooking apparent gains in collagen nitrogen by the Hartley-Hall method. Samples analyzed by the modified method of Lowry, Gilligan, and Katersky showed consistent losses of collagen on cooking. In fact, a total of 60 cooked samples have been analyzed by this method, all of which, when compared with the corresponding raw samples, have shown losses of collagen on cooking. Experience with samples containing less collagen than those reported in Table 1 has indicated that agreement between replicate samples is more difficult to attain as the level of collagen decreases.

Since collagen is, at present, a vaguely defined protein or group of proteins, it is hardly surprising that two quite different analytical methods give different results. The method of Hartley and Hall was proposed for the rapid analysis of meat, substituting the Waring blendor and the centrifuge for the more time-consuming ball mill and linen filter of earlier methods. By contrast, the method of Lowry, Gilligan, and Katersky was developed for the determination of collagen and elastin in small samples of tissues of different species.

SUMMARY

The collagen content of raw and cooked meat, and of purified steer-hide collagen has been determined by two methods. The results indicate that

the method of Lowry, Gilligan, and Katersky with slight modifications, is superior to that of Hartley and Hall for comparisons of the collagen content of raw and cooked meat, and that the Hartley-Hall method gives high results, especially for cooked meat. Results on the collagen content of purified steer-hide collagen determined by the two methods agreed within 5%.

LITERATURE CITED

1. Bergmann, M., and Stein, W. H. A new principle for the determination of amino acids, and its application to collagen and gelatin. *J. Biol. Chem.*, **128**, 217 (1939).
2. Block, R. J., and Bolling, D. *The Amino Acid Composition of Proteins and Foods. Analytical Methods and Results.* 2nd Ed., 1951, Charles C. Thomas, Springfield, Ill.
3. Bowes, J. H., and Kenten, R. H. The amino-acid composition and titration curve of collagen. *Biochem. J.*, **43**, 358 (1948).
4. Hartley, L. M., and Hall, J. L. Rapid determination of collagen in beef by Waring blendor and centrifuge technique. *Food Research* **14**, 195 (1949).
5. Lowry, O. H., Gilligan, D. R., and Katersky, E. M. The determination of collagen and elastin in tissues, with results obtained in various normal tissues from different species. *J. Biol. Chem.*, **139**, 795 (1941).

THE EFFECT OF INGREDIENT VARIATIONS ON THE QUALITY OF WHITE AND CHOCOLATE CAKES BAKED PRIOR TO FREEZING OR BAKED FROM FROZEN BATTERS[a]

ANDREA OVERMAN MACKEY, PATSY JONES, AND JOYCE DUNN
Department of Home Economics, Oregon Agricultural Experiment Station, Oregon State College, Corvallis, Oregon

(Manuscript received July 30, 1951)

The characteristics of freshly baked cakes may vary due to many factors, including the ingredients used, method of combining, baking pans, and oven temperatures. It may be anticipated that the quality of cakes held in frozen storage or baked from frozen batters will, likewise, be influenced by many of these factors. Differences in the kind or amount of ingredients used, though small enough to have little effect on the quality of fresh cake, might have a marked effect on cakes or batters which are stored for several months at freezing temperatures.

Few data have been reported dealing with the effect of ingredients on frozen cakes or batters. Graul and Lowe *(2)* found that the flavor and interior appearance of frozen cakes or cakes baked from frozen batters were less desirable when made with hydrogenated lard than when made with butter. Zaehringer and Mayfield *(5)* studied frozen storage of cakes and batters at 5,000 feet elevation. They used vegetable shortening, butter, and lard with tartrate baking powder, and vegetable shortening with sulfate-phosphate[b] baking powder. They concluded that only the vegetable shortening, sulfate-phosphate cakes and batters were satisfactory for frozen storage at 5,000 feet elevation.

The object of this study was to ascertain the effect of certain variation in ingredients and storage conditions on the quality of cakes when baked and held in frozen storage, or baked from frozen batters.

PROCEDURE

The study included white and chocolate cakes. They were varied as to ingredients used, and conditions of frozen storage. Some were baked before freezing while others were stored as batters and baked when removed from the locker. Cake quality was evaluated by means of palatability scores and volume measurements.

The study was carried out in three parts: 1, cakes baked before freezing to investigate the effect of different fats, pH values, and length of time in frozen storage; 2, batters, frozen to observe the effect of different types of baking powder, pH, fats, storage temperature, and storage time on the quality of cakes baked from these batters; and 3, batters, frozen to determine the effect of different levels of emulsifier and storage time on the quality of cakes baked from these batters.

Cakes baked before freezing. There were included 9 variations each of white and chocolate cakes, prepared with 3 different fats, 3 pH values, and tartrate baking powder. The fats were partially hydrogenated lard containing emulsifier and an antioxidant,

[a] Published as Technical Paper No. 698 with the approval of the Director of the Oregon Agricultural Experiment Station. Contribution of the Department of Home Economics.

[b] The term sulfate-phosphate baking powder was suggested by Chittick *(1)* for the combination of sodium aluminum sulfate and phosphate salts.

a vegetable shortening containing added emulsifier, and butter to which 5% glyceryl monostearate was added. The cakes were baked, then stored at 0°F.(−18°C.) up to 6 months for white cakes and 7 months for chocolate cakes.

Cakes baked from frozen batters for comparison of pH, fats, baking powders, and storage temperatures. In this group 12 variations each of white and chocolate batters were prepared using the 3 different fats described in the preceding paragraph. They were made with sulfate-phosphate and tartrate baking powders, and with and without added sodium bicarbonate. Preliminary trials had shown that batters containing added potassium acid tartrate did not rise appreciably when baked, even though frozen as short a time as 1 week at 0°F.(−18°C.); hence batters more acid than the basic formula were not included in this series. The batters were stored at 0°F.(−18°C.) up to 3 months, and at −30°F.(−34.5°C.) up to 6 months. They were baked when removed from the freezer.

Cakes baked from frozen batters for comparison of emulsifier level. The 6 variations each of chocolate and white batters were prepared with tartrate baking powder. Two fats, butter and a vegetable shortening which contained no emulsifier, were incorporated. Glyceryl monostearate was used at 0, 5, and 10% levels on a fat basis. These batters were stored at 0°F.(−18°C.) up to 3 months and were baked when removed from the freezer.

Methods used for all cakes. Moderately high sugar ratio formulas were used for both white and chocolate cakes, and the ingredients were combined with a modified conventional method of mixing. Then 120 g. of tartrate batter and 110 g. of sulfate-phosphate batter were weighed into individual 8-ounce round tin pans having slip covers. The pans were used as baking pans and for storing batters and baked cakes. Batters were baked at 374°F.(190°C.) for 19 minutes.

The 3 replications of all variables were prepared in random order. Variations in pH were accomplished by adding sodium bicarbonate or potassium acid tartrate, thus securing a more alkaline or a more acid reaction than that of the basic formula (Table 1). Cakes were designated as "alkaline," "acid," or "neutral," respectively.

For the determination of pH, 5 ml. of water was added to 10 g. of batter and 20 ml. of water to 10 g. of baked cake. The mixtures were blended quickly and thoroughly. Then 2 readings were made for each replication, the second after restirring the mixture.

At monthly or bimonthly intervals the batters and baked cakes were removed from the locker, allowed to come to room temperature, then weighed. The batters were baked and cooled. Cross sections were drawn and the cakes were scored. For scoring, the outside surfaces of baked cakes were cut away and only the interior of the cake was judged.

The index to volume was determined by measuring the cross section area of all cakes with a compensating polar planimeter *(5)*.

A panel of 5 judges scored the cakes for flavor staling, desirable moistness of crumb, and over-all desirability.

Analyses of variance were used for cake scores and volumes in testing the significance of difference of the various treatments. Analyses were computed for each storage period separately. An analysis could not be computed for the group of data as a whole because of unequal numbers of observations at different times. Following the analysis of variance, Tukey's method *(4)* was used in comparing cake means.

Trends throughout storage were determined by means of plotted data and the significance of differences at each storage period, Figure 1.

RESULTS

Weight changes of batters and baked cakes during frozen storage were small and showed no trends due to ingredients used. Baked cakes lost an average of 0.31 g. per cake weighing an average of 110 g. when put in the freezer. The weight loss of batters stored for 3 months at 0°F.(−18°C.) averaged 0.13 g., while those stored 6 months at −30°F.(−34.5°C.) averaged 0.24 g.

Average pH values for all cakes and batters are shown in Table 1. Baked cakes had higher pH values than the corresponding batters. Ex-

TABLE 1

Average pH values of chocolate and white cakes and batters made with 3 fats, 2 baking powders, and with added cream of tartar or sodium bicarbonate

	Variation in basic formula											
	No change in formula, "neutral"				Potassium acid tartrate added, "acid"				Sodium bicarbonate added, "alkaline"			
	Vegetable shortening	Lard	Butter	Average	Vegetable shortening	Lard	Butter	Average	Vegetable shortening	Lard	Butter	Average
	Tartrate baking powder											
White												
Batter	6.11	6.12	6.06	6.10	5.77	5.81	5.75	5.78	6.62	6.60	6.66	6.63
Baked	6.49	6.49	6.56	6.51	5.95	5.91	5.95	5.94	8.23	8.32	8.12	8.22
Chocolate												
Batter	5.93	6.00	5.96	5.96	5.75	5.72	5.68	5.72	6.61	6.67	6.51	6.60
Baked	6.35	6.34	6.34	6.35	5.98	5.98	5.95	5.97	7.99	8.02	7.88	7.96
	Sulfate-phosphate baking powder											
White												
Batter	6.62	6.72	6.52	6.63	.....	.....	.....	.....	6.96	6.98	6.83	6.92
Baked	7.02	7.01	6.91	6.98	.....	.....	.....	.....	8.75	8.68	8.46	8.63
Chocolate												
Batter	6.53	6.47	6.43	6.48	.....	.....	.....	.....	6.97	6.81	6.82	6.87
Baked	6.64	6.71	6.60	6.65	.....	.....	.....	.....	7.91	7.93	7.83	7.89

TABLE 2

Average cross section areas in square inches of cakes baked prior to freezing, then stored at 0°F.(—18°C.). Cakes were made with tartrate baking powder.

Storage time at °F.(—18°C.)	Kind of cake	Cake identification								
		Butter			Lard			Vegetable shortening		
		"Neutral"	"Alkaline"	"Acid"	"Neutral"	"Alkaline"	"Acid"	"Neutral"	"Alkaline"	"Acid"
months										
0	Chocolate	4.92	6.66	4.90	5.00	5.94	4.59	5.04	5.89	4.61
3	Chocolate	4.60	6.02	4.36	4.75	5.93	4.47	4.54	5.86	4.40
6	Chocolate	4.67	5.88	4.60	4.22	5.47	4.02	4.22	5.43	4.31
0	White	4.94	5.70	4.78	4.42	5.10	4.60	4.52	5.34	4.34
3	White	4.85	5.86	4.74	4.88	4.56	4.83	4.67	5.37	4.62
6	White	4.91	5.64	4.70	4.82	5.25	4.58	4.46	5.32	4.36

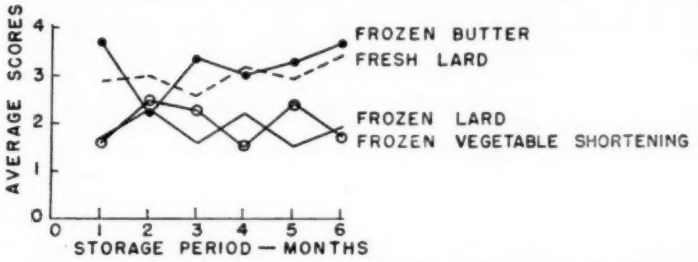


Figure 1. Average scores for moistness of white alkaline cakes made with different fats. Cakes were baked, then frozen and scored at monthly intervals up to 6 months. The frozen butter and fresh lard samples were significantly different from the frozen lard and frozen vegetable shortening samples in all tests except that conducted after 2 months' storage.

cept for chocolate cakes containing added sodium bicarbonate, all batters and cakes made with sulfate-phosphate baking powder were more alkaline than corresponding ones made with tartrate baking powder.

Volume measurements and palatability scores indicated that baked cakes responded differently to the freezing treatment than did batters.

Cakes baked prior to freezing. Frozen storage had little effect on the volumes of cakes that were baked before freezing. White cakes that were baked, then frozen and stored at 0°F.(−18°C.) up to 6 months showed no significant change in volume from freshly baked cakes of the same kind, whereas prebaked chocolate cakes showed a loss in volume which was significant, as indicated by the analysis of variance, but not sufficiently great to be of practical importance. The average cross section areas of cakes when freshly baked and stored up to 6 months at 0°F.(−18°C.) are shown in Table 2.

Flavor and desirability changes took place in some of the prebaked cakes during frozen storage. These changes were largely influenced by the fat used and by the length of time the cakes were stored. The pH made no apparent difference in these qualities. When made with butter, both white and chocolate cakes were judged to be most desirable throughout the storage period. Lard cakes developed most flavor staling; this staling became evident after 2 months for white cake and after 4 months for chocolate cake.

The quality of moistness was also influenced more by the fat used than by pH. White cakes made with butter were scored most moist regardless of pH. Chocolate "alkaline" cakes made with butter scored higher for moistness, whereas no difference due to the fat used was noted in the moistness of "acid" or "neutral" chocolate cakes.

When the cakes were removed from the pans, preparatory to scoring, a rancid odor was noted in the butter and lard cakes after 6 months of storage at 0°F.(−18°C.). This odor was more pronounced around the edges of the pan. The fat in the narrow rim of baked crust which clung to the pan, having a large surface area, developed rancidity faster than the fat in the cake. This odor was not detected by the judges, probably because all

crusts were removed before the cakes were scored. Once developed in the crust, however, the odor would soon penetrate the cake.

In addition to the development of off-odor and flavor staling in some cakes, all frozen cakes tended to change in texture, becoming crumbly rather than retaining the springy quality of freshly-baked cakes.

Frozen batters. The palatability of cakes baked from frozen batters was largely determined by the volume and texture of the cakes, though off-flavors were noted in lard and butter cakes baked from batters that had been stored 6 months at —30°F.(—34.5°C.). The volumes of cakes baked from frozen batters were influenced by the following variables: temperature at which the batters were stored; length of time batters were stored; kind of cake, whether chocolate or white; pH; type of baking powder; and emulsifier content.

In contrast to the negligible volume loss observed in cakes baked prior to freezing and stored at 0°F.(—18°C.), cakes baked from batters stored at 0°F.(—18°C.) were markedly smaller in volume and more compact in texture than cakes baked from freshly prepared batters of the same kind. The volume loss was most noticeable for cakes baked from chocolate batters, from batters made with tartrate baking powder, from batters having a "neutral" pH, and from batters containing no added emulsifier. The results are shown in Table 3.

When "neutral" in reaction, chocolate batters that had been stored for 1 month at 0°F.(—18°C.) tended to yield cakes that were undesirably compact. As batters were stored for increasing periods of time at 0°F.(—18°C.) the volumes of cakes baked from the batters continued to decrease and compactness of texture to increase, until after storage for 3 months, "neutral" chocolate batters frequently yielded cake failures. Corresponding white batters kept somewhat better, as indicated by cake volumes. The white cakes baked from "neutral" batters stored at 0°F. (—18°C.) for 1 month were considered good except for lard cakes which were less satisfactory. The cakes were increasingly compact as storage of the batters was prolonged, until after 3 months at 0°F.(—18°C.) the "neutral" white batters yielded cakes that were judged to be fair or poor, with some failures.

Cakes baked from frozen "alkaline" batters showed a percentage loss in volume which was approximately the same as that shown by the "neutral" batters. However, freshly prepared "alkaline" cakes were large, hence cakes of comparatively satisfactory volume were obtained from frozen "alkaline" batters even though the cakes were smaller than when freshly prepared. The chocolate "alkaline" batters made with sulfate-phosphate baking powder were more dependable than those made with tartrate baking powder from the viewpoint of yielding cakes of satisfactory volume and texture. When stored at 0°F.(—18°C.) for 3 months, all of this type of batter yielded satisfactory cakes, whereas a few of those made with tartrate baking powder yielded cake failures. The white "alkaline" batters made with either type of baking powder and stored up to 3 months at 0°F. yielded cakes of satisfactory volume, but poor cell structure.

Cakes baked from batters stored at —30°F.(—34.5°C.) tended to develop irregularities in texture rather than to show volume losses as storage

TABLE 3

Average cross section areas, in square inches, of cakes, freshly prepared and baked from frozen batters which were stored at 0°F.(—18°C.) and —30°F.(—34.5°C.)

Temperature	Storage time	Cake identification									
		Kind	Butter			Lard			Vegetable shortening		
			"Neutral"	"Alkaline"	"Acid"	"Neutral"	"Alkaline"	"Acid"	"Neutral"	"Alkaline"	"Acid"
Sulfate-phosphate baking powder											
°F.	*months*										
0(—18°C.)	0	Chocolate	4.34	5.79	.....	4.36	5.60	.....	3.94	5.43	.....
	1		3.85	5.31	.....	3.74	5.17	.....	3.77	5.49	.....
	2		3.43	4.99	.....	3.20	4.70	.....	3.38	4.56	.....
	3		2.98	4.69	.....	2.51	4.03	.....	2.46	4.07	.....
—30(—34.5°C.)	2		4.13	5.68	.....	3.87	5.38	.....	4.01	5.08	.....
	4		4.10	5.61	.....	4.00	5.30	.....	3.85	5.10	.....
	6		4.07	5.62	.....	4.02	5.05	.....	3.87	5.07	.....
0(—18°C.)	0	White	4.48	5.09	4.45	4.29	4.63	4.24	3.97	4.71	3.99
	1		4.08	4.57	.....	4.14	4.73	.....	4.16	4.57	.....
	2		4.07	4.53	.....	4.14	4.79	.....	4.16	4.72	.....
	3		3.72	4.38	.....	3.82	4.30	.....	3.75	4.40	.....
—30(—34.5°C.)	2		4.09	4.41	.....	4.16	4.76	.....	4.10	4.06	.....
	4		4.30	4.93	.....	4.15	4.60	.....	4.01	4.70	.....
	6		4.08	4.76	.....	4.08	4.55	.....	3.91	4.41	.....
Tartrate baking powder											
0(—18°C.)	0	Chocolate	4.92	6.66	4.90	5.00	5.94	4.59	5.04	5.89	4.61
	1		4.41	5.82	.....	4.01	4.81	.....	3.59	5.10	.....
	2		4.17	5.57	.....	3.01	4.27	.....	3.12	4.47	.....
	3		3.28	4.89	.....	2.64	3.26	.....	2.39	3.64	.....
—30(—34.5°C.)	2		4.94	6.12	.....	4.18	5.27	.....	4.16	5.48	.....
	4		4.69	5.89	.....	4.46	4.80	.....	4.05	5.18	.....
	6		4.85	5.86	.....	3.85	4.90	.....	3.77	4.94	.....
0(—18°C.)	0	White	4.94	5.70	4.78	4.42	5.10	4.60	4.52	5.34	4.34
	1		4.51	5.36	.....	4.80	5.15	.....	4.67	5.44	.....
	2		4.55	4.96	.....	4.61	5.11	.....	4.44	4.93	.....
	3		4.13	4.33	.....	4.12	4.62	.....	3.80	4.49	.....
—30(—34.5°C.)	2		4.52	5.55	.....	4.72	5.27	.....	4.51	5.62	.....
	4		4.75	5.43	.....	4.65	5.03	.....	4.52	5.41	.....
	6		4.76	5.50	.....	4.57	5.11	.....	4.65	5.30	.....

time of the batters progressed. Chocolate "neutral" batters stored at —30°F.(—34.5°C.) for 2 months or longer tended to yield cakes having compact moist streaks at the bottom. Chocolate "alkaline" batters stored up to 6 months at —30°F.(—34.5°C.) yielded cakes having good texture and volume.

The fats used had little effect on the volume of cakes baked from frozen batters. However, the fats incorporated did affect the texture and the

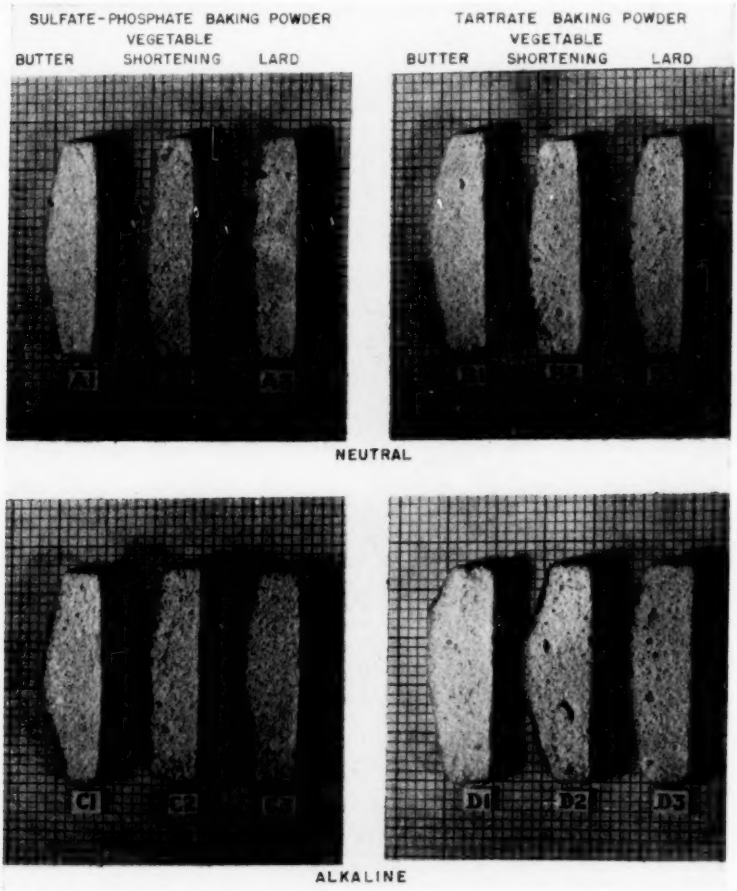


Figure 2. Volume and texture of cakes baked from white batters stored at —30°F. (—34.5°C.) for 6 months. Batters were prepared with 3 fats, 2 baking powders, and with and without added sodium bicarbonate. Code numbers indicate the following: A, sulfate-phosphate baking powder, "neutral"; B, tartrate baking powder, "neutral"; C, sulfate-phosphate baking powder, "alkaline"; D, tartrate baking powder, "alkaline"; 1, butter; 2, vegetable shortening; 3, lard.

flavor of some of these cakes. The white batters, both "neutral" and "alkaline" when made with butter yielded cakes of good texture throughout 6 months of storage at—30°F.(—34.5°C.) whereas the cell structure of cakes baked from similarly stored batters which were made with lard or vegetable shortening became increasingly coarse and irregular (Figure 2). In addition to texture changes, off-flavors developed when batters were stored a long time. This was noted especially in lard and butter cakes baked from batters that had been stored for 6 months at —30°F.(—34.5°C.).

As has been mentioned, a separate series of batters was frozen to study the effect of added glyceryl monostearate (Table 4).

TABLE 4

Average cross section areas in square inches, of cakes, freshly prepared and baked from frozen batters. Batters were made with tartrate baking powder, butter or hydrogenated vegetable shortening, with and without added emulsifier.

Storage time at 0°F. (—18°C.)	Kind	Cake identification					
		Butter			Hydrogenated vegetable oil		
		Glyceryl monostearate			Glyceryl monostearate		
		0%	5%	10%	0%	5%	10%
months							
0	Chocolate	4.14	5.01	5.59	4.55	5.06	5.17
1		4.45	4.58	4.92	3.12	4.59	4.96
2		2.95	4.35	4.51	2.58	4.47	4.53
3		2.49	4.13	4.47	2.60	4.26	4.30
0	White	4.63	5.26	5.52	4.63	4.89	5.50
1		4.30	4.57	4.84	4.32	4.35	5.03
2		4.04	4.73	4.73	4.32	4.48	5.07
3		3.47	4.15	4.55	3.88	3.82	4.44

When no emulsifier was used, the freshly prepared cakes, both white and chocolate, were close-grained in texture, fluffy in appearance, and springy to the touch. When 5% emulsifier was added, the cells were smaller and more uniformly distributed, and the cell walls were thinner. The internal appearance was fluffy and the cakes were springy to the touch. When 10% emulsifier was added, chocolate cakes were poor in internal structure, having thick cell walls, irregular cell distribution, and a moist rubbery appearance; the cut surfaces of white cakes were smooth and shiny rather than fluffy and velvety. These cakes tended to be brittle or crumbly rather than springy.

After storage for 3 months at 0°F.(—18°C.), the batters containing 10% glyceryl monostearate yielded cakes having somewhat better volume than those containing 5%. Cakes baked from white batters containing 10% emulsifier had good texture and satisfactory volume. At each emulsifier level those made with butter tended to have better cell structure than those made with vegetable shortening. The structure of chocolate cakes baked from frozen batters containing 10% emulsifier tended to improve as batters were stored. However, cakes baked from these batters were not as satisfactory in texture as the sulfate-phosphate "alkaline" cakes described above.

SUMMARY

Cakes baked prior to freezing had no appreciable volume change during 6 to 7 months of storage at 0°F.(−18°C.). Palatability changes were influenced chiefly by the fat incorporated, butter cakes scoring highest throughout frozen storage. The texture of cakes stored for 6 months was noticeably crumbly as compared with the springy texture of freshly baked cake.

Cakes baked from batters stored at 0°F.(−18°C.) up to 3 months were smaller in volume than cakes baked from freshly prepared batters of the same kind. The volume loss was most noticeable for cakes baked from chocolate batters, from batters made with tartrate baking powder, from batters having a "neutral" pH, and from batters containing no added emulsifier. The most satisfactory cakes obtained from batters stored for 3 months at 0°F.(−18°C.) were the chocolate "alkaline" cake made with sulphate-phosphate baking powder, and the white butter cake containing 10% glyceryl monostearate.

Cakes baked from batters stored at −30°F.(−34.5°C.) up to 6 months had good volume. The texture of white cakes baked from these batters showed uniform cell distribution when made with butter, whereas, if made with vegetable shortening or lard the texture was more coarse and irregular. A rancid flavor was noted in lard and butter cakes baked from batters that had been stored for 6 months at −30°F.(−34.5°C.).

LITERATURE CITED

1. Chittick, J. R. Graphic baking powder chart. Jaques Manufacturing Co., Chicago, Ill.
2. Graul, L. S., and Lowe, B. How storage affects frozen cakes and batters. *Food Inds.*, **19**, 330 (1947).
3. Platt, W., and Kratz, P. O. Measuring and recording some characteristics of test sponge cakes. *Cereal Chem.*, **10**, 73 (1933).
4. Tukey, John W., Comparing individual means in the analysis of variance. *Biometrics Bull.*, 99 (1949).
5. Zaehringer, Mary V., and Mayfield, Helen L. The effect of leavening and shortening combinations on the frozen storage of cakes and cake batters prepared at high altitude. *Food Technol.*, **5**, 151 (1951).

REDUCTION OF TRIMETHYLAMINE OXIDE IN DOGFISH FLESH[a]

R. PAUL ELLIOTT[b]
Fiskeridirektoratets Kjemisk-Tekniske Forskningsinstitutt, Bergen, Norway

(Manuscript received August 30, 1951)

Trimethylamine oxide (TMAO) was first demonstrated in marine fish by Suwa *(14, 15)*. Poller and Linneweh *(11)* showed that it was reduced to trimethylamine (TMA) by bacteria when the fish spoiled. Beatty and Gibbons *(2)* were the first to find that TMA production could be used as a measure of fish decomposition, and Beatty *(1)* showed that TMAO is the precursor of at least 94% of the TMA formed in spoiling cod muscle press juice. The bacterial enzyme causing the reduction was first described by Tarr *(16, 17)*. TMA has been shown to be one of the principal contributors to the spoilage odors of various marine teleost fishes by investigators too numerous to mention. On the other hand, the characteristic odor of spoilage in elasmobranch fishes is that of ammonia formed by the breakdown of the large quantities of urea present in the flesh. That such breakdown is caused partly by autolytic enzymes in the muscle but mostly by the action of bacteria has been shown by Ferguson-Wood *(6)*. Various workers have found that the flesh of elasmobranch fishes generally contains 2 to 5 times as much TMAO as that of the marine teleosts. These data have been summarized by Reay, Cutting, and Shewan *(12)*. On the other hand, it was shown by Shewan *(13)*, Reay, Cutting, and Shewan *(12)*, and by Hjorth-Hansen and Bakken *(9)* that despite the larger amount of TMAO present, TMA forms slowly in shark flesh and does not exceed the amounts formed in spoiling teleost species.

The object of the present study was to confirm the existence of and to determine the reason for the phenomenon that TMAO is not quickly and completely reduced to TMA in the spoilage of elasmobranch fishes.

EXPERIMENTAL

Spoilage of dogfish meat. Four pigged dogfish (*Squalus acanthias* L.) averaging 71 cm. in length, were received in excellent fresh condition in the round. They were butchered with a sterile knife, and the carcasses, exclusive of head, viscera, fins, and tail, were minced 4 times in a sterile meat grinder with stirring between grindings. Replicate portions of the homogenous mass, weighing about 250 g. each, were placed in sterile glass jars with loose-fitting glass lids. The jars were wrapped in paper and stored at 0°C. At 3-day intervals, the contents of single jars were taken as samples for analysis.

A 150-g. portion of the sample prepared and stored as described above was placed in a sterile vertical-type blending jar. To this was added 150 ml. of chilled sterile sea water. The mixture was blended for 3 minutes, and 20 g. of the blend was weighed into a bottle containing 80 ml. of chilled sterile sea water. This gave a 1 to 10 dilution.

[a]This investigation was supported by a Fulbright fellowship granted by the U. S. Department of State, Division of Exchange of Persons.

[b]Present address: U. S. Food and Drug Administration, Federal Building, San Francisco, California.

Numbers of viable bacteria were determined on this portion by the plate count method of Elliott *(5)*.

From the remaining blend, 200 g. was weighed into a beaker, and 200 ml. of distilled water was added. The pH was adjusted to 5.2 with a measured volume of normal HCl, and the total volume of the mixture brought to 500 ml., which made a 1 to 5 dilution of the original sample. It was heated to 70°C., held at that temperature for 5 minutes, cooled, and filtered. The nitrogenous bases were determined in the filtrate. This method of preparing the extract was suggested by Hjorth-Hansen and Bakken *(9)*.

On the extract or a dilution of it with distilled water, nitrogenous bases were estimated by the microdiffusion method of Conway *(4)*. TMA, TMAO, ammonia, and urea were also determined. For convenience in calculation, standard solutions of HCl and of NaOH were adjusted to exactly 0.0286 *N*. for use in the determinations. All analyses and blanks were made in triplicate. In all cases 1-ml. portions of standard HCl were used in the inner chambers of the Conway dishes. In the outer chambers, a variety of reagents were used. For TMA,[c] 1 ml. of formalin was added to 1 ml. of the extract, followed, after mixing, by 1 ml. of saturated K_2CO_3. For TMAO, 3 drops of 15% titanous sulphate *(8)* were added to reduce the oxide, and after 10 minutes the TMA was determined. The oxide was found by difference. Total volatile base was determined by adding 1 ml. of saturated K_2CO_3 to 1 ml. of the extract. Ammonia was taken as the difference between total volatile base and TMA. Urea was determined using 0.5 ml. of urease phosphate solution to 1 ml. of the extract as descriebd by Hawk, Oser, and Summerson *(7)* to break down the urea to ammonia. After a reaction time of 15 minutes, 1 ml. of saturated K_2CO_3 was added. Total volatile base was subtracted to obtain the figure for urea. All dishes were incubated overnight at room temperature before being titrated.

Results of the storage test on minced dogfish flesh are shown in Figure 1. The pigged dogfish is like other elasmobranch species in that TMA does not form in quantities commensurate with the amount of TMAO present in the flesh. When minced dogfish flesh was stored at 0°C. in sterile jars, the TMA content increased slowly in the first days, but after 12 days of storage, there was no further increase in TMA nor further decrease in TMAO. During this time the total bacteria count had risen and fallen again to nearly its original value. After 6 days of storage, slight traces of ammonia could be detected by smell. By the ninth day, the odor was very strong and masked all other odors. The experiment was extended to 28 days of storage, by which time the remaining samples had begun to mold. Triamineoxidase-producing bacteria were found in the fish at all stages of spoilage. This discounts the possibility that such bacteria died off before the reduction of TMAO could go to completion.

Shewan *(13)* found that on prolonged storage in ice, the TMAO in the flesh of dogfish carcasses slowly disappeared. As Shewan suggested, this could have been partially due to the leaching action of the melting ice. TMAO did not disappear from the minced flesh held out of contact with melting ice in the present experiments.

The sum of ammonia and urea nitrogen was a constant during the entire course of the experiment. This shows that the principal source of the ammonia is the urea present in the muscle. The pH rose to 9 as spoilage reached an advanced stage, whereas the pH of the flesh of most teleost species rises to only about 7.8 in advanced spoilage.

[c] The method used for determination of TMA is that of Beatty and Gibbons *(2)*. Actually, dimethylamine is also distilled over under the conditions of the determination. However, the amount of dimethylamine formed during the spoilage of dogfish is very small *(13)*, and was therefore ignored.

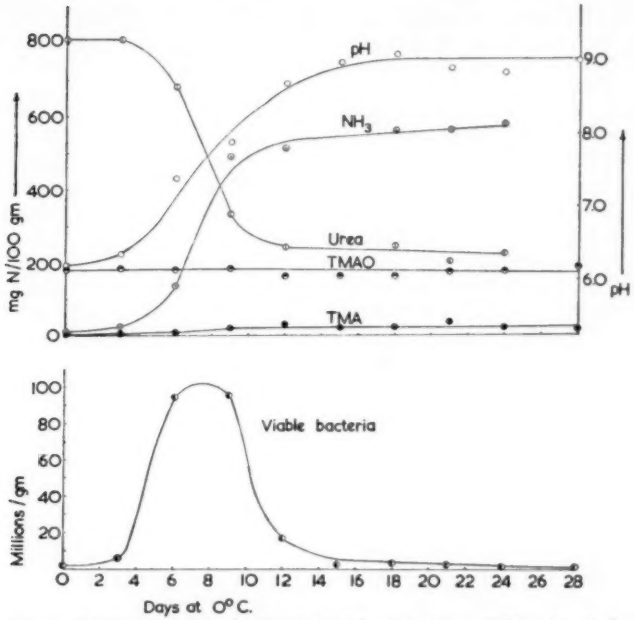


Figure 1. Spoilage history of minced dogfish (Squalus acanthias L.) flesh stored in loosely-closed sterile glass jars.

Effect of urea on TMAO reduction. In this case, 900 ml. of muscle press juice was obtained from pre-rigor cod (*Gadus morrhua* L.). This juice was separated into 2 lots. To one lot was added 1.6% urea, the other was left unaltered as a control. Both samples were stored at 0°C. in cotton-stoppered Erlenmeyer flasks and analyzed for nitrogenous bases at 3-day intervals. Preparation of the nitrogenous extract was the same as described above except that blending was omitted and only 10-ml. quantities were used. Analyses were made as previously described.

Results of this experiment are shown in Figure 2. The reduction of TMAO in cod muscle press juice containing 1.6% urea stopped completely when the breakdown of urea had produced a relatively high ammonia concentration and a resultant high pH. The TMAO in the unaltered control was reduced quantitatively to completion within 9 days. This experiment implied that either the ammonium ion or the hydroxyl ion might be responsible for the phenomenon being investigated.

Effect of ammonium hydroxide and sodium hydroxide on TMAO reduction. The preceding experiment was repeated, but the cod muscle press juice was divided into 3 lots instead of 2. To Lot 1 was added small amounts of concentrated NH_4OH each day to bring the ammonia content to that found at the same stage of spoilage in the preceding experiment in which urea was added at the start. A small amount of concentrated NaOH was added to the second lot of juice each day to bring the pH to exactly the same as that occurring in Lot 1. Lot 3 was retained as an unaltered control. Extracts were prepared from 10-ml. samples, and analyses for nitrogenous bases were made as described above.

Results of this experiment are shown in Figure 3. Both NH_4OH and NaOH have the ability to stop the reaction when only part of the TMAO is reduced. This indicates that high pH is the cause for the failure of TMAO to be reduced to completion in the cod muscle press juice containing urea, and discounts the possibility that the ammonium ion alone is the cause. It is interesting to note that when ammonia was added the pH rise was slightly greater than that occurring when urea was added, even though the ammonia concentration was the same. This difference may be partly due to the neutralizing effect of carbonic acid during the breakdown of urea.

Effect of neutralizing shark muscle press juice during spoilage. Juice from the muscle of the pigged dogfish was prepared and divided into 2 lots. Each day, Lot 1 was neutralized to pH 7.2 with concentrated HCl when the pH had risen above that figure, whereas Lot 2 was allowed to spoil naturally as a control. Analyses for nitrogenous bases were made at 3-day intervals as described previously.

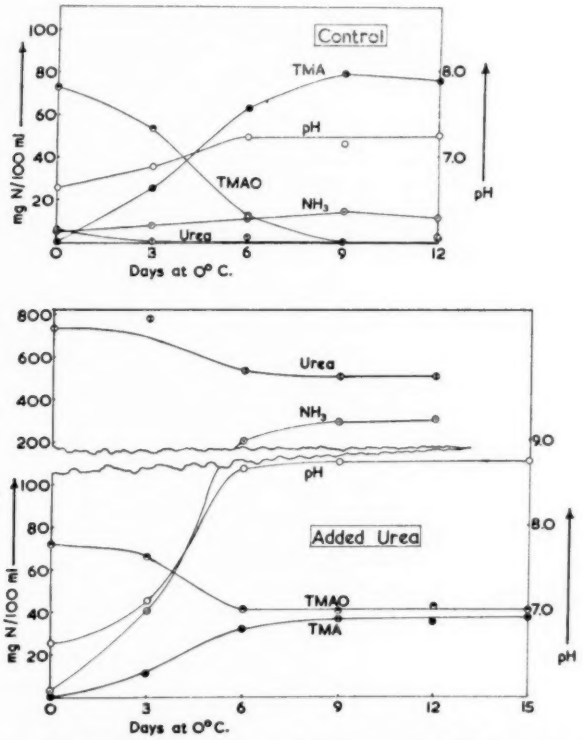


Figure 2. Effect of added urea on the reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) in cod muscle press juice. Ammonia was formed from the breakdown of urea as the juice spoiled.

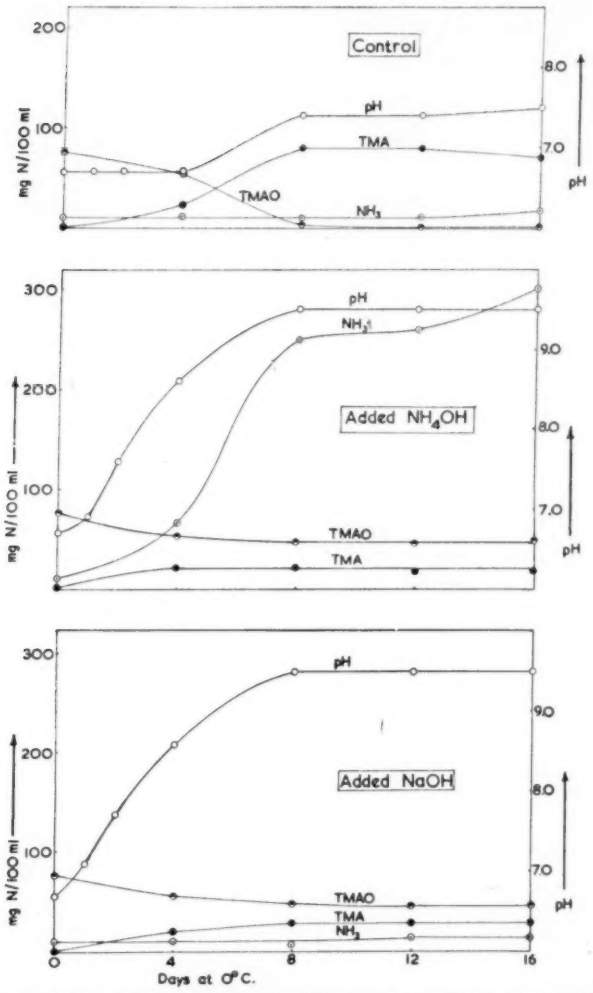


Figure 3. Effect of added NH$_4$OH and NaOH on the reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) in cod muscle press juice.

Results of this experiment are shown in Figure 4. In dogfish muscle press juice whose pH was kept in the zone 7.2 to 8.0 by the daily addition of HCl, the reduction of TMAO progressed to completion in 18 days. In the unaltered control in which the pH rose to 9.2, reduction ceased completely when only about 30% of the TMAO had been reduced. These

results are taken to be final proof that high pH is the reason why TMAO is not completely reduced in the spoilage of dogfish flesh.

Optimum pH of the enzyme triamineoxidase. Although Castell and Snow *(3)* studied the effect of pH of the enzyme that reduces TMAO they did not extend their experiments into the highly alkaline range which occurs during shark-meat spoilage. To test the effect of pH values up to 9, an experiment following the method described by Castell and Snow was undertaken. TMAO and sodium acetate were dissolved in 10-ml. portions of full-strength buffer solutions prepared as described by Lang *(10)*. Phosphate buffer was used at pH 6.6, 7.0, 7.6, and 8.0; borate buffer at 8.0, 8.4, 8.6, and 9.0. Then 2 ml. of a 3% NaCl suspension of washed cells of a 3-day bacterial culture isolated from thoroughly spoiled dogfish meat was used as a source of the enzyme. This suspension, when diluted 1 to 10, gave 43% transmittance in an Evelyn colorimeter. By earlier tests, the culture had been found to produce triamineoxidase. The TMAO used in the experiment was prepared by mixing TMA and hydrogen peroxide solutions in the cold. After the mixture was refrigerated overnight, the water was

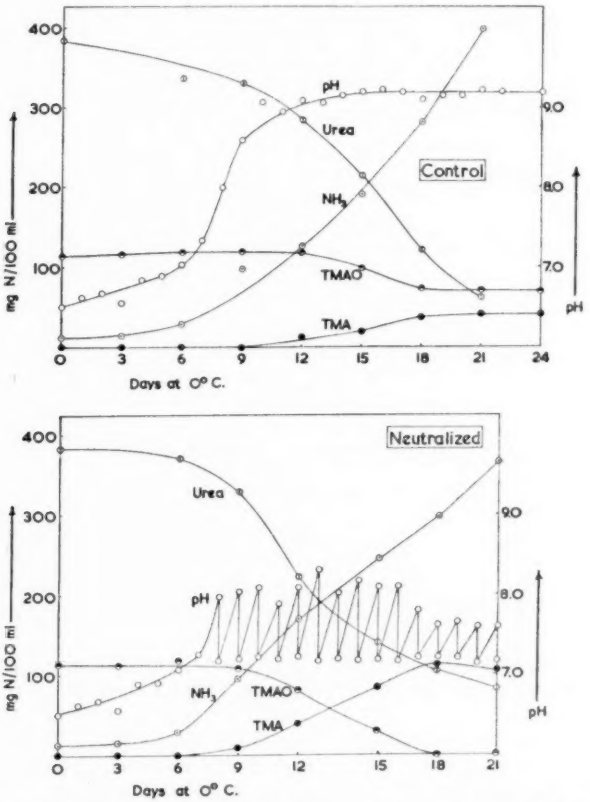


Figure 4. Effect of neutralization on the reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) in spoiling dogfish muscle press juice.

distilled off under high vacuum and the resultant crystals were twice dissolved in ethyl alcohol and recrystallized under vacuum. The enzyme study was conducted at 30°C. in unevacuated but closed Thunberg tubes. Tubes were opened momentarily for pipetting of samples when analyses for TMA were made at 2, 4, and 6 hours. It was found that the pH values of those solutions near the optimum pH for the enzyme rose slightly as a result of production of excess TMA. In no case was this rise greater than 0.2 pH unit during the 6-hour period. The pH values of the solutions at 8.4, 8.6, and 9.0 did not change. TMAO was determined at 6 hours to indicate how much TMA evaporated during the course of the experiment.

Results are shown in Figure 5. The optimum pH for triamineoxidase was found to be in the vicinity of 7.2 to 7.4. This confirms the results of Castell and Snow *(3)*. At pH values above and below this, activity

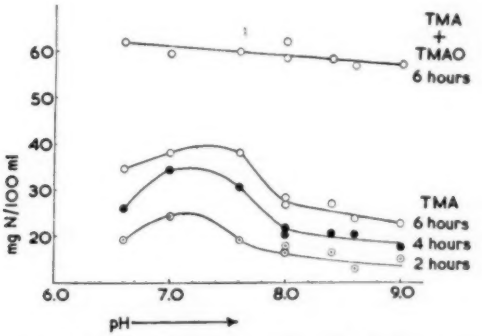


Figure 5. Optimum pH of triamineoxidase. The experiment was conducted at 30°C. with solutions of trimethylamine oxide (TMAO) and sodium acetate dissolved in full strength buffers. The enzyme was added as a heavy suspension of washed cells from a bacterial culture known to reduce TMAO to trimethylamine (TMA).

decreased progressively. There was a small amount of evaporation at the higher pH values as indicated by the drop in the sum of TMA plus TMAO after 6 hours had elapsed. But this evaporation was not high enough to account for the low values obtained for TMA in the alkaline solutions.

Effect of high pH on prolonged activity of triamineoxidase. The previously described experiments have demonstrated that high pH is the reason for the phenomenon that TMAO is not reduced to completion in shark meat (Figures 3 and 4). Yet considerable TMA was formed in buffered solutions even at pH 9, though the quantity was less than that at the optimum pH (Figure 5).

To investigate this apparent discrepancy, a second enzyme study was undertaken using only 2 unevacuated, closed Thunberg tubes. Conditions were the same as those described in the previous experiment. One tube was buffered with full-strength phosphate buffer at pH 7.2 and the other with full-strength borate buffer at pH 9.0. (In the previously described enzyme study, borate and phosphate buffers at pH 8.0 had been found to have equal effect on triamineoxidase.) To avoid the problem of TMA evaporation, the disappearance of the non-volatile TMAO was taken as a criterion of enzyme activity. Production of TMA in the solution buffered at pH 7.2 caused the pH to rise gradually, despite the buffer. Twice during the experiment, the pH was readjusted to pH 7.2 with concentrated HCl. The pH of the solution buffered at 9.0 did not change.

Results of this experiment are shown in Figure 6. When the pH of the substrate was kept near the optimum for the enzyme, the reduction of TMAO eventually went to completion; but when the TMA produced raised the pH to near 9, the reaction ceased, and proceeded only when the pH was brought nearer the optimum. When the pH of the substrate began at and remained near 9, the reduction began slowly, reached the same rate as that at the lower pH, then ceased completely when only part of the TMAO had been reduced.

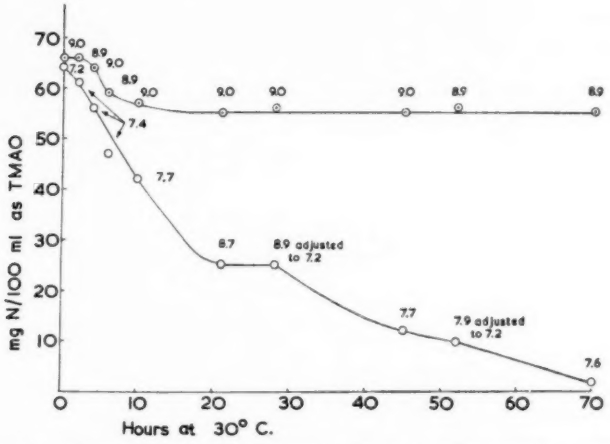


Figure 6. Effect of pH on the prolonged action of triamineoxidase in removing trimethylamine oxide (TMAO) from buffered solutions containing sodium acetate. Production of trimethylamine at pH 7.2 raised the pH despite the buffer. The solution was neutralized with concentrated hydrochloric acid at the two points indicated. Figures on the curves are pH values of the solutions when samples were taken.

This experiment explains why TMA formation in spoiling shark flesh reaches a certain level, then stops completely despite the abundance of the unaltered precursor. In short, there is apparently a quantitative relationship between hydroxyl-ion concentration and TMA concentration that affects the activity of triamineoxidase. The exact relationship could not be determined from the available data.

SUMMARY

The trimethylamine oxide (TMAO) present in minced pigged dogfish muscle was not reduced to completion during the spoilage process at 0°C. on storage for 28 days. Reduction ceased after about 12 days of storage when only ⅙ of the TMAO had been reduced to trimethylamine (TMA).

In the muscle juice of the cod, however, TMAO was completely reduced to TMA in 9 days at 0°C. By adding urea, NH_4OH, or NaOH to cod muscle press juice, reduction of TMAO to TMA was caused to cease when only part of the TMAO had been reduced.

On keeping pigged dogfish muscle juice close to the optimum pH for triamineoxidase by the addition of hydrochloric acid, the TMAO present was found to be reduced to TMA quantitatively to completion in 18 days, whereas in the unaltered juice, reduction ceased when only part of the TMAO had been reduced.

These experiments indicate that the reduction of TMAO in the pigged dogfish is prevented from going to completion because of the high pH caused when urea breaks down to ammonia when spoilage begins. The sum of ammonia nitrogen and urea nitrogen was a constant during the spoilage period.

The optimum pH of triamineoxidase was found to be from 7.2 to 7.4. In substrates of higher pH, triamineoxidase activity was less, but some TMA was produced, even at pH 9. When the pH of a substrate was at or near the optimum for triamineoxidase and was not allowed to approach 9, TMAO was reduced to completion by the enzyme. When production of TMA raised the pH to near 9, activity ceased, but proceeded when the solution was neutralized. In a substrate buffered at pH 9, triamineoxidase activity started slowly, reached a rate equal to that at the optimum pH, then ceased abruptly.

These experiments explain why TMA formation in spoiling shark flesh reaches a certain level, then stops completely despite the abundance of unaltered TMAO. The urea present in the flesh is broken down, principally by bacterial action, to form ammonia. This occurs relatively quickly, before the TMAO reduction has had a chance to gain momentum. The ammonia raises the pH to approximately 9, at which level only a part of the available TMAO will become reduced to TMA by the action of triamineoxidase. At this high pH the balance between TMAO and TMA is believed to have a quantitative relationship with the hydroxyl ion. The exact nature of this relationship could not be determined from the available data.

Acknowledgment

The author wishes to thank the personnel of the Fiskeridirektoratets Kjemisk-Tekniske Forskningsinstitutt for their courtesy in lending facilities and obtaining materials used in the study. He wishes to thank especially Miss Elise Sparre of that organization for her assistance in some of the laboratory analyses.

LITERATURE CITED

1. Beatty, S. A. Studies on fish spoilage. II. The origin of trimethylamine produced during the spoilage of cod muscle press juice. *J. Fisheries Research Board Can.*, **4**, 63 (1938).
2. Beatty, S. A., and Gibbons, N. E. Measurement of spoilage in fish. *J. Biol. Board Can.*, **3**, 77 (1936).
3. Castell, C. H., and Snow, J. M. The effect of pH on the enzymatic reduction of trimethylamine oxide. *J. Fisheries Research Board Can.*, **7**, 561 (1949).
4. Conway, Edward J. *Microdiffusion Analysis and Volumetric Error.* 1947, Crosby Lockwood and Son Ltd., London.
5. Elliott, R. Paul. Preliminary study of total bacterial plate count method for fishery products. *Com. Fisheries Rev.*, **10** (11), 11 (1948).
6. Ferguson-Wood, E. J. The bacteriology of shark spoilage. *Australian J. Marine Fresh Water Research*, **1**, 129 (1950). Com. Fisheries Abstracts, **3** (11), 11 (1950).
7. Hawk, Philip B., Oser, Bernard L., and Summerson, William H. *Practical Physiological Chemistry.* 12th Ed., 1947, J. and A. Churchill, Ltd., Portman Square, London.
8. Hjorth-Hansen, Sverre. Metode for bestemelse av trimetylaminoksyd. *Fiskeridirektorat. Skrifter,* Serie Undersøkelser ved Statens Fiskeriforsøksstasjon (P. O. Box 494, Bergen, Norway), Vol. 2, *in print.* (1951).

9. HJORTH-HANSEN, SVERRE, AND BAKKEN, KAARE. Undersøkelser over analysemetoder for ammoniakk og metylaminer i fisk. Fiskeridirektorat. Skrifter, Serie Undersøkelser ved Statens Fiskeriforsøksstasjon (P. O. Box 494, Bergen, Norway), **1**, No. 6 (1947).

10. LANG, NORBERT ADOLPH. *Handbook of Chemistry.* 6th Ed., 1946, Handbook Publishers, Inc., Sandusky, Ohio.

11. POLLER, K., AND LINNEWEH, W. The occurrence of trimethylamine oxide in *Clupea harengus. Ber.*, **59**, 1362 (1926), (*Chem. Abstracts* **20**, 2882[9] [1926]).

12. REAY, G. A., CUTTING, C. L., AND SHEWAN, J. M. The nation's food. VI. Fish as food. II. The chemical composition of fish. *J. Soc. Chem. Ind. (London)*, **62**, 77 (1943).

13. SHEWAN, J. M. Spoilage of fish. Dep. Sci. Ind. Research (Brit.). *Rept. Food Invest. Board*, **1938**, 79 (1939).

14. SUWA, A. Untersuchungen über die Organextrakte der Selachier. I. Die Muskelextraktstoffe des Dornhais *(Acanthias vulgaris). Arch. ges. Physiol. (Pflügers)*, **128**, 421 (1909).

15. SUWA, A. Untersuchungen über die Organextrakte der Selachier. II. Über das aus den Muskelextraktstoffen des Dornhais gewonnene Trimethylaminoxyd. *Arch. ges. Physiol. (Pflügers)* **129**, 231 (1909).

16. TARR, H. L. A. The bacterial reduction of trimethylamine oxide to trimethylamine. *J. Fisheries Research Board Can.*, **4**, 367 (1939).

17. TARR, H. L. A. Specificity of triamineoxidase. *J. Fisheries Research Board Can.*, **5**, 187 (1940).

EXPLORATORY EXPERIMENTS TO IDENTIFY CHEMICAL REACTIONS CAUSING FLAVOR DETERIORATION DURING STORAGE OF CANNED ORANGE JUICE. I. INCOMPATIBILITY OF PEEL-OIL CONSTITUENTS WITH THE ACID JUICE[a]

J. S. BLAIR, EDITH M. GODAR, J. E. MASTERS, AND D. W. RIESTER
Research and Technical Department, American Can Company, Maywood, Illinois

(Manuscript received September 12, 1951)

It is quite apparent that the flavor deterioration which takes place during the unrefrigerated storage of canned orange juice is greater than that which is encountered during the similar storage of canned non-citrous fruit products. Observations made in this laboratory with canned Florida Valencia orange juice stored at any temperature above 70°F.(21°C.) may be described by saying that the predominant off-flavor which develops under such conditions is "terebinthine" in character. The terebinthine factor is acrid and camphoraceous, and is often most clearly apparent as an after-flavor, perceived after swallowing the juice. Similar juice stored at temperatures which never exceed 70°F.(21°C.) also depart in time from a desirable fresh flavor, but in this case the off-flavor has a complex and variable quality which may be named "stale" for purposes of discussion, and which is conspicuous in such samples because at the lower temperature the terebinthine factor does not become dominant. Such empirical observations indicate quite clearly that the over-all degradation in flavor during storage involves a large number of different chemical reactions, differing in regard to the temperature coefficient of reaction rate.

It has been postulated in the past that flavor degradation is due to microbiological activity *(30)* or to changes in nitrogen compounds *(19)*, or to products resulting from a postulated initial oxidation of juice components. The primary oxidation products are not themselves off-flavored but are thought to constitute an unstable system which leads to the ultimate production, from the fatty components of the juice, of substances which impart to the flavor a rancid quality (20). It is probably fair to say that the proponents of these hypotheses were seeking explanations of the stale, rather than the terebinthine, flavor factors.

In the present paper evidence is presented which shows that the terebinthine flavor factor, specifically, is due to a chemical incompatibility of the terpenic components of the peel-oil (including D-limonene, its principal constituent) with the acid juice. The reactions involved are the acid-catalyzed hydration-dehydration (ACHD) reactions of the terpenes, which are discussed in any of the standard monographs on the subject *(10, 24)*. Evidence will be presented to show that the rates of these reactions are a

[a]Some of the material presented in this paper was discussed with the members of the Florida Section of the Institute of Food Technologists at its meetings on March 17, 1947 and on March 30, 1950.

function of pH level, but are fundamentally independent of the presence or absence of oxygen. In view of this evidence it is a safe conclusion that even if it were possible to exclude oxygen completely during the canning of orange juice, the absence of oxygen would not prevent the development of the terebinthine flavor in the canned juice during storage above 70°F. (21°C.). The intensity of this flavor after storage can be mitigated by raising the pH level of the juice a few tenths of a pH unit before canning, but this involves in the fresh juice a loss of natural tartness which is undesirable, suggesting somewhat the flavor of overripe oranges.

Work in this laboratory as well as the work of others *(5, 6)* has shown that the terebinthine factor does not develop in peel-oil-free canned orange juice. Unfortunately, fresh orange juice completely devoid of peel-oil is relatively insipid, although it does have, to a varying degree, a pleasant fruity character usually suggestive of a mixture of apple and apricot juices. But it lacks the distinctive orange-like character which, in normal juice, results from the presence of a small amount of peel-oil. In the extraction of orange juice for canning, the procedures and equipment used in present practice automatically incorporate peel-oil to an extent ranging ordinarily from about 50 to about 300 p.p.m. These limits have been well established as those characterizing a juice of desirable flavor.

Recognition of the fact that development of the terebinthine flavor during storage is due to this incompatibility of the peel-oil with the acid juice does not seem to appear anywhere in the technical literature of orange juice except in the patent of Kunz *(15)*. Kunz recognized that "the fruit acid contained in the citrus fruits . . . may vigorously attack, modify or destroy the flavoring principle derived from the rind of the fruits . . .", causing "the development of a foreign disagreeable odor in the syrup, which is commonly referred to as rancid, terebenthine, or of the nature of turpentine." Kunz was interested in the preparation of fruit syrups, and recommended the preparation of 2 separate syrups, one from the juice and one from the flavoring principle of the rind, and mixing them only at the time of consumption. To confirm the indications of the analytical work of the present investigation, canned peel-oil-free orange juice after warm storage has been flavored at the time of tasting by addition of a small amount of orange oil stored separately, giving a product free from the terebinthine flavor at least, and also superior in general flavor to the control lot which was prepared by adding the same oil to the same peel-oil-free juice before canning.

The analytical evidence presented herein for the validity of the ACHD reactions as the cause of the terebinthine flavor is based largely on infra-red absorption spectrometry of the oil phases distilled from canned orange juice after storage under various conditions. The same spectrograms also give evidence of the hydrolysis of esters during storage of the juice, producing acids which are to some extent oil-soluble. This part of the infra-red evidence has been confirmed by direct measurement of an increase in oil-soluble acid associated with increased temperature of storage. However, this situation is a complex one. Not all of the esters which are being hydrolyzed to give oil-soluble acids are peel-oil constituents; some are constituents of the fruit pulp. And not all of the oil-soluble acids which are produced are malodorous; some are odorless. The contribution made to off-flavor by certain of these acids doubtless comprises a part of the "stale" flavor as discussed above. Only to the extent that the antecedent esters are peel-oil constituents, therefore, may we say that this part of the stale flavor is due to incompatibility of peel-oil with the juice.

Among the other contributors to the complex stale effect may be listed a phenol, having a clove-like odor, which is produced during storage from some water-soluble constituent of the fruit. Certain sulfur compounds, also deriving apparently from the fruit pulp rather than the peel-oil, appear to be very important contributors to this stale effect. Our study of these factors is incomplete and a more detailed discussion of them will be reserved for a later communication.

The Physical State of the Oil Dispersed in Orange Juice

Work in this laboratory, described herein, has established the fact that D-limonene, which constitutes about 90% of the peel-oil and is itself an indispensable component of true orange flavor, is soluble in water to the extent of only about 7 p.p.m. It is evident, therefore, that in orange juice the peel-oil exists largely as a discrete oil phase, undissolved in water. This is to be contrasted with the situation existing in non-citrous fruit juices such as those of the apple *(22, 29)* or peach *(21)* wherein the flavorful essences are undoubtedly present entirely in water solution. Although orange peel-oil is less dense than water, it was shown by Smith *(25)* that when orange juice is centrifuged, a considerable part of the oil is present in the gelatinous phase which is thrown down in this procedure. This observation has been confirmed in the present work. It seems probable, in fact, that within the usual range of concentration practically all the oil exists as an adsorbed layer on the colloidally dispersed solids of the juice. On centrifuging, the coarser solids, with a part of the oil, are precipitated; while the finer solids with their adsorbed oil remain in suspension.

Approach to the Problem

In modern canning procedures the time during which the juice is hot before canning has been minimized, and as a result the freshly canned juice has a flavor little inferior to that of the raw material. Serious flavor degradation is encountered only during storage, and its dependence on the temperature of storage has been amply demonstrated. Simply stated, the colder the storage the better the flavor.

Therefore, in the present work, the initial approach was to place packs of Florida Valencia orange juice in controlled storage at 45°, 70°, and 95°F.(7°, 21°, and 35°C.), with the hope that analytical means could be found to give us clues as to the chemical differences developing in storage. Some of these chemical differences, correlated with storage temperature, could be expected to be significant in explanation of the flavor differences which soon became obvious. The first step in analysis was distillation at atmospheric pressure in a special oil-trap described below, which was designed to render the oil phase of the distillate readily available for examination. This oil phase was a convenient sample for examination by infra-red spectrophotometry, which has enabled much of the storage change to be characterized by means of experiments involving only 1.5 or 2.0 liters of juice. The off-flavor developing in canned orange juice, complex and variable though it is, is largely a matter of odor and can be perceived by the nose before the juice is taken into the mouth. This odor is to a considerable extent epitomized by the odor of the distilled oil phase,

which assures us that the analytical differences encountered in the distilled oil phase are pertinent to the flavor problem.

However, it has become apparent that the composition of this distilled oil phase is not identical with that of the oil phase actually existing in the juice at room temperature; and any reaction products, whether originating from peel-oil or from fruit-pulp precursors, will upon distillation distribute themselves between the 2 phases (oil and water) of the distillate according to their solubilities under the conditions existing in the oil trap, rather than according to their origin. Actually, the evidence of centrifugation experiments, described in this paper, indicates that the malodorous components as they exist in the can are for the most part in water solution, even though upon distillation they are largely caught and held in solution in the distilled oil phase. This merely means that during the re-cycling distillation their concentration in the boiling aqueous phase is small.

The Acid-Catalyzed Hydration-Dehydration (ACHD) Reactions of the Terpenes

D-Limonene, as an olefinic hydrocarbon, becomes reactive in an acid environment, giving rise by reversible reactions to a whole system of hydrocarbons, alcohols, diols or "glycols," and ethers as represented in Figure 1. It will be observed that the introduction of the oxygen which is present in certain of these compounds has come about by the chemical addition of water, and not by oxidation. Speaking generally, it is the alcohols and particularly the diols which account for the decrease in yield of recoverable oil which has been found to take place during the warm storage of orange juice or of a canned limonene-citrate buffer system, while the terebinthine odor is attributable primarily to the ethers (cineoles) and to a lesser extent to the terpene hydrocarbons which are isomeric with limonene but are not so bland in odor as the starting material.

The predominant initial reaction is the chemical addition of water, or hydration, of D-limonene to D-alpha-terpineol, an alcohol whose water-solubility is given by standard reference as about 2 g. per liter. It is evident, therefore, that this substance must dissolve in the aqueous phase almost completely and as fast as it is formed by the chemical interaction at the oil-water interface, although as explained above it will be present in the oil phase obtained by distillation. The optical rotation of alpha-terpineol is practically as high as that of limonene itself, so that its presence in the distilled oil phase is not manifested by a significant decrease in the measured rotation. Its presence is clearly demonstrable, however, by several strong and characteristic absorption bands in the infra-red spectrum. In fact, since alpha-terpineol is always the predominant product of ACHD change under any conditions commonly met with in the storage of canned orange juice, its characteristic absorption bands are a convenient index of the extent of ACHD change under ordinary conditions, and the general congruency of the bands found in the oil phase distilled from canned orange juice after warm storage with the bands found in known limonene-terpineol mixtures is the most obvious proof of the validity of the ACHD hypothesis. Alpha-terpineol is often used as a scent for soap; its odor is sometimes described as "like lilac" and is not extremely pungent. The substance must be added to orange juice in

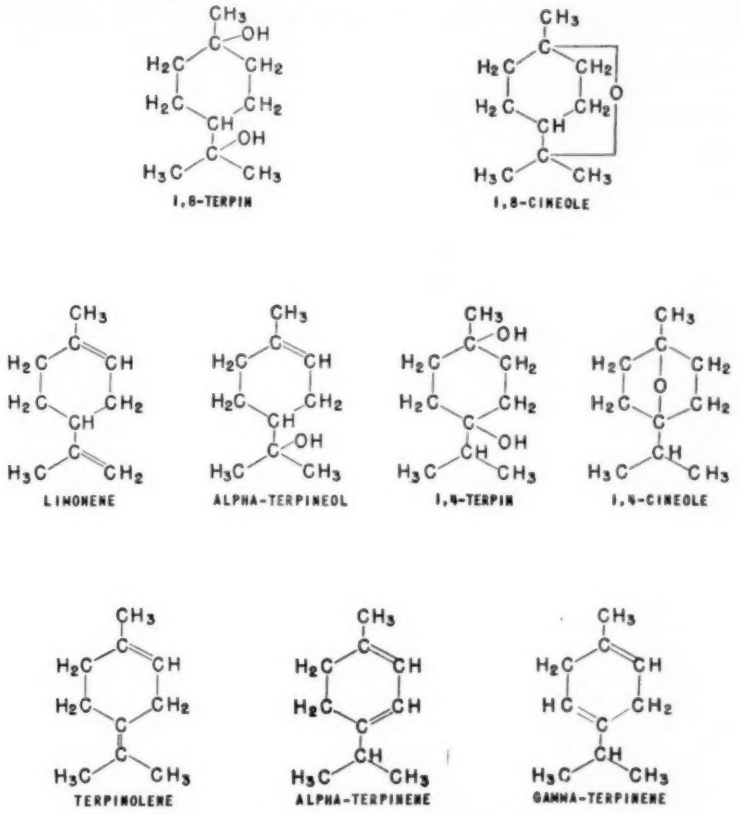


Figure 1. Structural formulas of ACHD components.

a concentration of about 10 p.p.m. before it has a perceptible effect on flavor.

A further hydration may now take place, forming a diol, 1,8-terpin, which is still more soluble in water (3.6 g. per liter as the crystalline hydrate) than is terpineol. A saturated water solution of terpin hydrate, although practically odorless, has a somewhat bitter taste. Direct experiment has shown that terpin is not volatile with steam. Limonene distilled from a saturated aqueous solution of terpin hydrate exhibits an infra-red spectrum identical with that of the same limonene distilled from water alone. The formation of this substance in the storage of canned orange juice is not manifested by any effect on the properties of the distilled oil phase, nor upon odor, but it is manifested by a decrease in the yield of recoverable oil upon distillation.

Terpin then undergoes an acid-catalyzed dehydration to form an ether, 1,8-cineole. This substance occurs in eucalyptus oil and has an extremely pungent camphoraceous odor. Standard references give its water solubility at room temperature as about 3.5 g. per liter, decreasing with increase in temperature. This substance can be well purified and it exhibits several characteristic infra-red absorption bands. Our evidence indicates that both 1,8-cineole and its isomer, 1,4-cineole, are formed during the warm storage of canned orange juice. There are certain characteristic differences in the infra-red spectra of the 2 cineoles. Admittedly we are dealing here with trace amounts of these substances, and we cannot use a good infra-red solvent free from absorption bands of its own, but must accept limonene, admixed with terpineol, as our solvent. In the oils from orange juice, the cineoles ordinarily produce only slight deviations in the absorption curves. However, when canned limonene-citrate buffer systems are stored at 120°F. (49°C.) or at even higher temperatures, the bands of the cineoles are readily perceptible. The odor of 1,4-cineole is fully as pungent as that of 1,8-cineole and appears to be less agreeable (more acrid) than that of the 1,8-isomer. The 1,4-cineole can be detected by its effect on flavor when added to orange juice at a concentration less than 1 p.p.m. Like terpineol, the cineoles exist in the can largely in water solution, but are caught and held by the oil phase upon distillation.

Alpha-terpinene is a terpene hydrocarbon for which a good analytical method exists *(8)*, based on the fact that as a conjugated system it forms a crystalline adduct with maleic anhydride. A sample prepared by us and and known by this means to be at least 95% pure has shown that alpha-terpinene has a strong and characteristic absorption band at a wavelength of 12.10 μ. This band shows up quite definitely, not only after warm storage of a limonene-citrate pack but also after long storage at 95°F. (35°C.) of orange juice itself. Alpha-terpinene appears to have an odor more pungent than that of limonene but not so offensive as that of some other products of ACHD change. It is optically inactive, and therefore its presence in the distilled oil phase can contribute to a decrease in the optical rotation thereof. Its solubility in water is unknown; it may well be as low as that of limonene. Nevertheless, the hydrocarbon is probably present largely in water solution in the aged orange juice, in view of its low concentration and in view of the fact that saturation of water with limonene does not abolish the solvent power of the water for other hydrocarbons.

Terpinolene is an optically inactive, chemically unstable terpene hydrocarbon which, as the literature shows, is very difficult to isolate in the pure state. We are not sure of the complete purity of any of the samples of terpinolene prepared by us as yet, but samples that should consist primarily of terpinolene have a strongly acrid odor which resembles a component of the terebinthine flavor factor. Direct evidence for the presence of terpinolene in the oil phase distilled from canned orange juice stored under any ordinary conditions is difficult to obtain from the infra-red spectrum. As the structural formulas of Figure 1 show, the conversion of limonene to terpinolene is the conversion of an isopropylene to an isopropylidene group. The analytical distinction between these 2 groups has

long been a vexatious problem of general importance in terpene chemistry, and an experimental approach to it based on infra-red spectrophotometry was the subject of a recent paper by Barnard et al. *(2)*. As these authors point out, the isopropylene group exhibits a strong and characteristic band at 11.23 μ (which is shown very strongly by limonene), but the isopropylidene group, as present in terpinolene, shows neither this band nor any other band of corresponding intensity. The infra-red method is therefore a very sensitive detector of the isopropylene group, but the presence of isopropylidene compounds in low concentration readily escapes detection. The same generalization was found by Frank and Berry *(7)* to apply in their infra-red study of the isomerization of 8(9)-p-menthene (isopropenyl) to 4(8)-p-menthene (isopropylidene). We are forced to postulate, therefore, that terpinolene is probably present in warm-stored canned orange juice in a concentration sufficient to influence odor but too low to be objectively demonstrable by our present analytical means. But since the formation of alpha-terpinene and of 1,4-cineole from limonene requires more far-reaching ACHD change than does the formation of terpinolene, evidence for their presence in any system constitutes justification for postulating the presence of terpinolene also.

Gamma-terpinene is included in Figure 1 because it is said to be formed concurrently in any system in which alpha-terpinene has been formed by ACHD change. Its boiling-point approximates that of terpinolene so that it may often be a contaminant of the latter. Certain preliminary evidence as to the formation of gamma-terpinene by ACHD change, and concerning an infra-red band characterizing the substance, will be mentioned in the experimental part of this paper.

SOME CONSIDERATIONS PERTAINING TO THE ACHD REACTIONS

General. Although the literature of terpene chemistry supports in a qualitative way the interrelations discussed in the preceding section, it is unfortunately true that almost nothing is known as to various important quantitative aspects of these relationships. It is interesting to consider what is known, or may be guessed at, as to the effect of various factors on the equilibrium state ultimately attained in a system initially consisting of limonene dispersed in a citrate buffer solution. A low proportion of limonene relative to the buffer should of course favor a high degree of conversion to the hydrated, water-soluble products. Temperature should affect not only the rate of reaction but also the equilibrium state, and there are indications that high temperature favors dehydration while a low temperature favors a high proportion of hydrated products in the equilibrium ultimately attained. The pH level certainly affects the reaction rate; the extent to which it may affect equilibrium is uncertain. In this connection it may be expected that alpha-terpinene, a conjugated system, would be among the more stable end-products. It has been observed that this hydrocarbon shows little tendency to rearrange to other terpenes under the influence of acids *(24)*. Some preliminary experiments with limonene-citrate buffer systems, which will not be described further in the present paper, indicate that the presence of sugar influences the ACHD

changes in the direction of inhibiting hydration and favoring dehydration, thus changing the relative proportions of the substances listed in Figure 1.

Influence of oxygen. In the classical investigations wherein the interactions of the terpenes with acid were worked out, no particular precautions were taken to exclude atmospheric oxygen, and no consideration was given to the possibility that oxygen might fundamentally affect the course and rate of these reactions. In the experimental part of this paper an experiment with a canned limonene-citrate buffer system is described, the results of which show that a relatively extreme variation in oxygen content produced no observable effect on the ACHD change encountered as a result of heating for 7 hours in boiling water. This shows that oxygen is in no way a fundamental factor in regard to ACHD change, and it is a conservative conclusion that the development of terebinthine off-flavor during warm storage could not be prevented or even significantly retarded by the complete exclusion of oxygen, if that were possible in the commercial packing of orange juice.

On the other hand, there are indications that under special conditions oxygen may produce trace effects superimposed upon the ACHD changes. While oxygen enclosed in a can of acid product is rapidly consumed at higher temperatures by reduction at the acid-metal interface *(11, 23)*, at low temperatures there should be greater opportunity for oxygen to react with constituents of the fruit. In cold storage the products to be expected from oxidation of limonene are certain odorless water-soluble products (diols and triols) which, however slow to form, are quite stable in the cold. This may be a partial explanation for the decrease in yield of recoverable oil which we have observed, in a limited number of cases, to take place during the protracted frozen storage of orange juice. The products of autoxidation of limonene tend to peptize the dispersion of limonene in water, as was evident in the experimentation to determine the solubility of limonene in water (see Experimental Methods), and as we have observed to a marked extent in the distillation of limonene-carvone mixtures in the oil-trap. It is quite possible, therefore, that the yield of oil recoverable after frozen storage is lowered more because of this physical property than because of actual chemical loss caused by the slight amount of oxidation involved.

The recent findings of Kefford, McKenzie, and Thompson *(12)* are pertinent in this connection. These authors were able to demonstrate that reduction of the initial oxygen content of canned orange juice to a very low level had no effect on the flavor deterioration in storage at 86°F. (30°C.), and only a slight effect at 68°F.(20°C.). The low oxygen content of their packs was proved by a polarographic method *(16)*, by means of which the relative persistence of oxygen in low-temperature storage [41°F. (5°C.)] was also demonstrated.

In accordance with an interesting generalization of Wallach *(26)*, the diols and triols referred to should undergo acid-catalyzed dehydration at subsequent higher temperatures to form cymene and a substance of carbonyl function (apparently carvenone, $C_{10}H_{16}O$; not carvone, $C_{10}H_{14}O$). During the course of this work an experiment, which will not be described in detail, was carried out in which pure limonene was thoroughly autoxi-

dized before packing with citrate, and in this case traces both of cymene and of substance of carbonyl function could be detected in the infra-red spectra after warm storage. The initial phases of ACHD change were also expedited in the pre-oxidized sample (probably because of the peptizing effect referred to above), although the unoxidized control sample appeared to "catch up" later and the 2 samples were much alike at the end of the experiment. But the important point is that to get such effects, even at the trace levels encountered, it was necessary to establish initially a degree of oxidation of limonene that would be highly improbable in the canning of orange juice. This is in accord with the fact that in none of our experiments with canned orange juice itself have we been able to detect cymene in the distilled oil phase, although infra-red absorption bands exist which seem to constitute characteristic and fairly delicate tests for its presence.

Effects due to the heterogeneous character of the reaction system. The very low solubility of limonene in water shows that the rate of initial ACHD reaction must be largely a function of the extent of the mutual surface between oil and aqueous phases as they exist in the canned juice, rather than a function of the total oil content. Therefore, it is not necessarily to be expected that the terebinthine flavor factor, after a reasonably short period of warm storage, would be worse in a juice of high oil content (say 200 p.p.m.) than in a juice of low oil content (say 50 p.p.m.). In fact, it may well be that for a time a high oil content may serve to disguise the terebinthine flavor to some extent, as indeed Curl and Veldhuis *(6)* have observed. This situation would not be expected, theoretically, if the oil, as it exists in the can, were completely dissolved in the aqueous phase.

EXPERIMENTAL METHODS AND RESULTS

Trap for Recovery of Distilled Oil Phase

The apparatus (Figure 2) used for recovery and measurement of the distilled oil phase was similar in principle to that of Bartholomew and Sinclair *(4)* but with the modification that the distilled aqueous phase was returned to the boiling flask. The 2-liter or 3-liter boiling flask was affixed to the trap at the 29/42 ST joint, *A*. The vapors rose through the vapor-tube *V* and were condensed on the finger condenser *C*, which was an extension of the 45/50 ST joint *B*. The condensing chamber was open to the atmosphere at *G*. The aqueous condensate returned to the flask through the return tube *R*, which established level *L*, above which the globule of distilled oil phase remained at *O* throughout the distillation. At the end of the distillation, the condenser was lifted at *B* and washed down with a jet from a wash-bottle, then replaced. The oil globule was then transferred to the vertical tube under *D* by blowing through a rubber tube attached at *G*. The trap was then removed from the boiling flask and connected to a leveling bulb (not shown) by attachment at *A*. Water was run from the leveling bulb until it began to raise the level of the oil. Outlet *G* was then closed and the water level carefully raised further to cause the oil to enter the calibrated 2-mm. capillary measuring tube *M*, through the 24/40 ST joint *D*, and stopcock *E*. Just prior to raising the oil, both *D* and *E* were lubricated with water, but the capillary part of *M* was kept dry. After getting the oil into *M*, the stopcock *E* was closed so as to hold the oil column steady while its length in the tube was being measured. This stopcock was then reopened and the oil run up into reservoir *R*, whence it could be transferred by means of a hypodermic syringe to a micropolarimeter tube, an IR cell, or other apparatus as desired.

Bartholomew and Sinclair distilled for 75 minutes at a rate of 100 ml. aqueous distillate each 15 minutes. In the present work the distillation was carried out at about the same rate, but for 30 minutes only, since the hazard of incomplete recovery was considered less important than the hazard of chemical change during distillation. Fur-

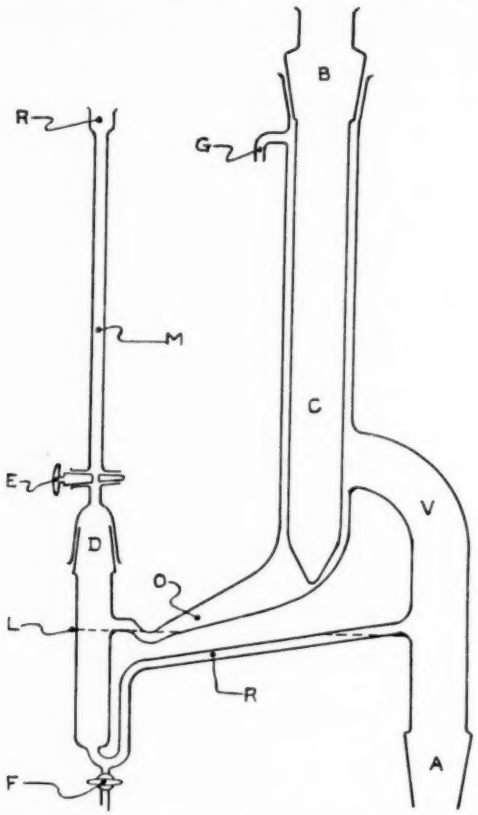


Figure 2. Oil trap apparatus used for the recovery and measurement of the distilled oil phase.

ther to minimize the latter hazard, in our later work the pH level of the juice samples was raised to pH 5.0 before distillation. It was found by direct experiment that distillation of juice at the natural pH level of about 3.5 yielded oil showing slightly greater ACHD change (IR evidence of increased terpineol) than did distillation of the same juice at pH 5.0. A further increase to pH 7.0 was avoided in work with juice, because esters are hydrolyzed more at pH 7.0 than at pH 5.0. However, in working up the limonene-citrate buffer packs, in which esters are absent, many of the distillations were carried out at pH 7.0. The terpene hydrocarbon is unaffected by an alkaline environment.

In Table 1 are presented the results of a series of experiments in which weighed amounts of freshly-purified limonene were distilled from 1 liter of water, or of canned peel-oil-free (POF) orange juice previously brought to pH 5.0. The oranges used in obtaining this juice were hand-peeled and surface-treated with potassium permanganate to destroy clinging peel-oil *(26)*. The yield of oil in distilling this juice alone was about 5 mg. per liter.

TABLE 1

Recovery of limonene from water and from peel-oil-free orange juice

Expt. No.	Distilled from	Limonene taken	Limonene recovered	Limonene not recovered
		mg.	*mg.*	*mg.*
1	Water	675	649	26
2	Water	670	638	32
3	Water	676	645	31
4	Water	703	681	22
5	POF juice	701	689	12
6	POF juice	687	666	21
7	POF juice	663	637	26
8	POF juice	679	663	16

It is believed that due to vapor being carried out at *G* by the vigorous outrush of air which takes place at that time, the principal loss of oil is incurred when boiling first starts. To obtain recoveries as good as those given in this table, it is necessary that the cooling water be really cool, that the glass surfaces be cleaned just before the distillation with a detergent and brushing, and that the collected oil be moved upward into the measuring capillary very slowly and carefully to ensure that no visible oil droplets are left adhering to the lower glass walls. Although the apparatus was always carefully cleaned, it is possible that the recovery data presented elsewhere in this paper are subject to somewhat greater error, in some cases, than those of Table 1. For one thing, the recoveries had to be carried out in more of a hurry in order to do further work with the oil, and in the summertime the cooling water may not always have been as cool as it should have been. We consider that the apparatus has been of value in giving oil samples conveniently for the qualitative work which is described herein, and is capable of further improvement to give better measurements of oil yield.

Stopcock *F* is being used in some of our work to enable the aqueous distillate to be scrubbed with carbon tetrachloride before allowing it to return to the boiling flask. The solvent is pipetted in through *D* at the start, and the solution run out through *F* for infra-red examination after the distillation is completed.

Solubility of Limonene in Water

During the course of this work a number of attempts were made to determine the solubility of limonene in water by weighing an excess of limonene into a liter of water and measuring the undissolved excess in the calibrated 2-mm. capillary tube used in connection with the apparatus described above. The earlier experiments were valueless; a stable emulsion was obtained and much of the undissolved limonene would not rise to be measured. Trouble of this kind was finally obviated by strict exclusion of air and by using limonene which was alkali-treated and steam-distilled (see below) immediately before use. The solubility vessel was made from 50 mm. Pyrex tubing about 70 cm. long, equipped with a 24/40 ST neck at one end, with stopcocks at both ends to permit the maintenance of a nitrogen atmosphere. Somewhat more than a liter of distilled water was boiled to eliminate dissolved air, and the boiling water poured into the vessel, previously filled with nitrogen, against a current of effluent nitrogen. The water was cooled and then run out down to a 1-liter calibration on the tube, under a

nitrogen blanket. The freshly purified limonene was weighed in and the tube capped, both against effluent nitrogen. After agitation for at least 24 hours the tube was clamped in a vertical position, at prevailing room temperature. Under these conditions all of the oil phase seems to rise to the top and the solution appears clear.

However, even under these conditions, the results obtained by running the undissolved oil up into the measuring tube by admitting water at the bottom from a leveling tube indicated a solubility of about 50 p.p.m. It was suspected that this value was grossly high, because of various losses of limonene occurring during the protracted experiment.

Therefore, recourse was had to an infra-red method. Here 1 liter of the clear saturated solution, obtained by the method just described, was distilled in the apparatus described in the preceding section, through a pipetted 0.5-ml. portion of mineral oil. The depth of the infra-red absorption band at 11.23 microns was measured for this solution, as compared with the absorption of the solvent. A number of standardizing curves were then obtained by distilling various weighed amounts of limonene from a liter of water through 0.5 ml. of mineral oil in the same manner. Perhaps some limonene in all cases fails to reach the mineral oil or remain in solution in it, but this makes no difference if these factors are constant.

Upon the basis of such experiments we can say that the true solubility of limonene in water in the neighborhood of 25°C. is much lower than that indicated by the earlier method. It appears to be lower than 10 p.p.m., higher than 5 p.p.m., and close to 7 p.p.m.

Standard Samples Used for Comparisons in the Infra-red Work (Curves 3A and 3B on Figure 3)

A Beckman IR-2 infra-red spectrophotometer in connection with a Brown recording potentiometer was used in these experiments. A 0.1-mm. cell spacer was used throughout. The energy curve of the source, using a salt block in the light path, is the uppermost curve in Figure 3, and is a legitimate reference for all the spectra. For the purpose of qualitative identification of trace components, the presentation of facsimile reproductions of portions of the original curves was considered to be preferable to the presentation of any type of derived curves.

Limonene. Orange oil was fractionated through a Podbielniak Hyper-Cal fractionating column (13 mm. × 36 in.) under a reduced pressure of nitrogen, to give limonene fractions which were then further purified with methanolic KOH. In a typical case 100 ml. of a fraction, boiling at 68°F.(20°C.) at 18 mm. of mercury and having an observed rotation in a 10-cm. tube of 102.1°, was shaken with 150 ml. of a 5% solution of KOH in anhydrous methanol, and allowed to stand overnight at room temperature. A yellow color developed in the limonene-poor upper phase, which was discarded. The colorless lower phase was washed with water and steam-distilled immediately before use, having then an observed rotation of 103.8°. Every set of curves in Figure 3 includes a curve obtained with a sample of limonene so purified.

Alpha-Terpineol (Curves 3A). This substance was crystallized from Eastman's P-403 alpha-terpineol by holding it at 70°F.(21°C.) after seeding. Without further purification it melted at 30-32°C. (literature, 35°C.). The phenylurethane melted at 110.5°C. (literature, 109.5°C.) after 1 recrystallization from aqueous ethanol. Two concentrations of terpineol in limonene are represented by infra-red absorption curves in Figure 3A. These concentrations are those of synthetic mixtures which were then distilled from water in the oil-trap. The proportions of terpineol to limonene in the distilled oil phases actually examined may differ somewhat, of course, from those of the samples as weighed out. Nevertheless, assuming constant conditions of distillation, the proportions as weighed out should be the proper "yardstick" by which to measure the ratio of terpineol to limonene in any sample, as of orange juice, taken for analysis.

Caprylic Acid (Curves 3B, band No. 1). Paragon's No. 5465 n-caprylic acid was fractionated under a reduced pressure of nitrogen. The fraction boiling at 119-120°C.

at 8 mm. Hg. pressure was used to make the standard solutions for the glass powder chromatography, and as a standard for the infra-red work. The absorption band shown in Curves 3B was obtained with a 3.5% solution of caprylic acid in limonene, after steam-distillation in the trap as described above. This band is that which is characteristic of the carboxyl group.

1,4-Cineole (Curves 3B, bands No. 2). The standard sample was obtained from 1,4-cineole 50% (Marbro Industries) by fractionation in the Podbielniak column under nitrogen. The fraction boiling at atmospheric pressure at 170.5-172.0°C., n_D^{25} 1.4502, was treated repeatedly with aqueous permanganate until this reagent was no longer reduced, giving a material having n_D^{25} 1.4467 (literature, 1.4479). The cineole thus obtained was extracted with a 50% aqueous solution of resorcinol, as recommended by Austerweil *(1)* for the elimination of any 1,8-cineole that might have been present. The material was then washed with water and steam-distilled. The 3 characteristic bands shown were obtained with an approximately 4% solution in limonene. The band at 9.0 μ is that which is characteristic of the ether group, but the bands at 8.25 and 12.0 μ are also useful in distinguishing this substance from 1,8-cineole.

1,8-Cineole (Curves 3B, bands No. 3). This material (boiling at 172°C. uncorrected), was prepared from Paragon's No. 1287 cineole by exhaustive oxidation of impurities with permanganate, followed by fractionation in a Claisen flask. A sample of Fritsche's U.S.P. eucalyptol, after similar treatment, boiled at 172.5°C. (uncorrected). After steam distillation the IR spectra of the 2 samples were almost perfectly congruent. The 3 bands shown were obtained with a 3.6% solution in limonene; the ether band being at 9.2 μ and the other two at 8.1 and 11.8 μ.

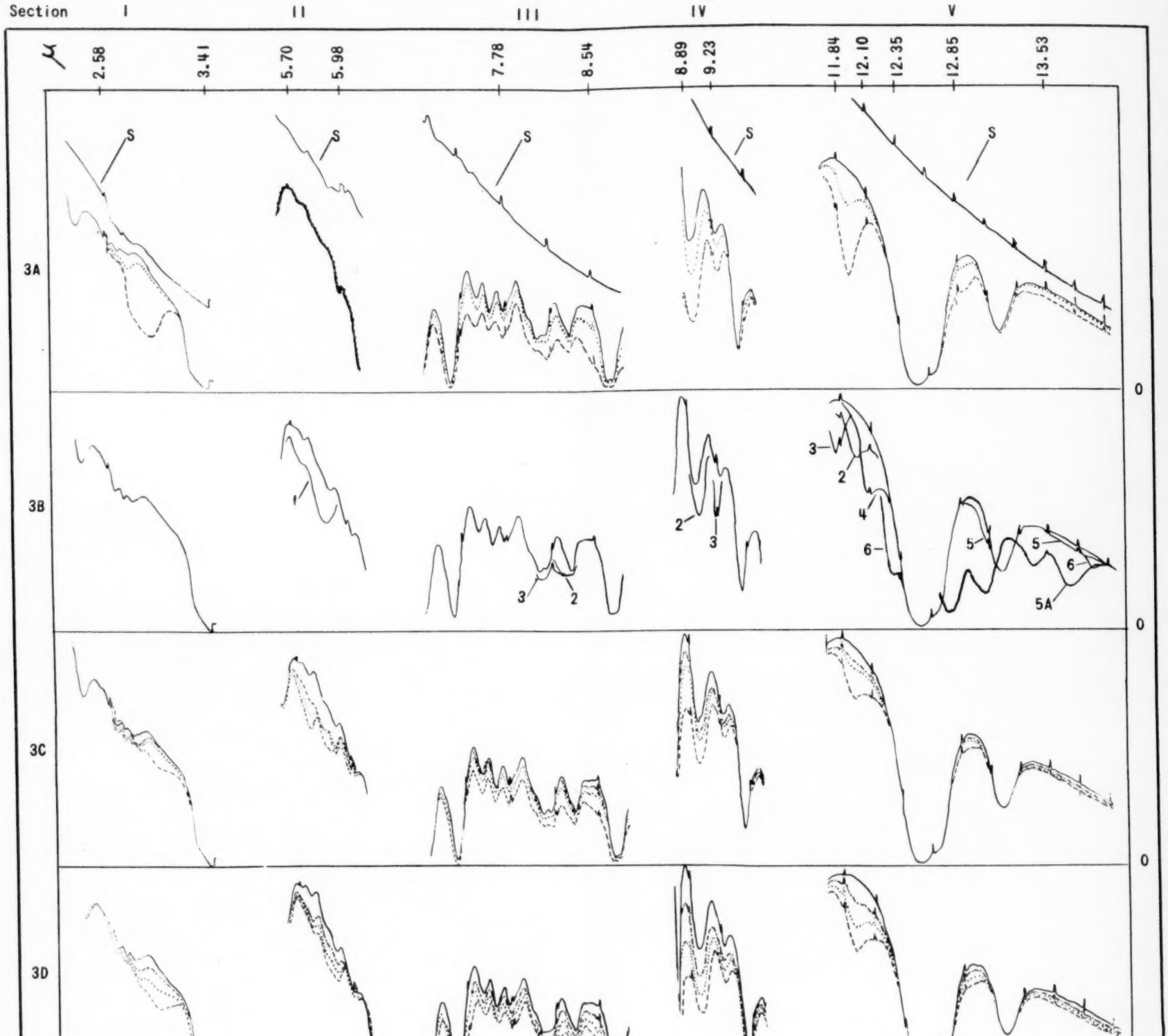
Alpha-terpinene (Curves 3B, band No. 4). The sample was prepared by Wallach's procedure *(27)*, in which alpha-terpineol was refluxed with 50% aqueous oxalic acid for 5 hours. The mixture was steam distilled and the oil phase dried and fractionated through the Podbielniak column under a reduced pressure of nitrogen. Fraction 3, boiling at 65°C. at 19 mm. Hg. pressure, assayed 97% for alpha-terpinene by the maleic anhydride method of Gascoigne *(8)*. The melting-point of adduct-anhydride was 62°C. (literature 61°C.). The absorption band at 12.1 μ shown in Curves 3B was obtained with a 3.1% solution in limonene, after steam distillation as described above. This absorption is very intense and is believed to be very characteristic for this substance.

"Terpinolene" (Curves 3B, bands No. 5). The sample was prepared by a procedure also given by Wallach *(27)*, in which alpha-terpineol was refluxed with aqueous oxalic acid for 30 minutes. The oil phase was separated and steam-distilled, then dried and fractionated through the Podbielniak column under reduced nitrogen pressure. Fraction 10, boiling at 77-78°C. at 16 mm. Hg., was used as a 9% solution in limonene to give the two broad and relatively weak bands shown in Curves 3B as bands No. 5, while the absorption of the same terpinolene fraction, undiluted by limonene, is shown as bands No. 5A. The results of some very recent work lead us to ascribe the bands at 13.08, 13.38, and 13.67 μ to terpinolene, but the band at 12.78 μ is believed to be due to the presence of gamma-terpinene as a major impurity.

p-Cymene (Curves 3B, bands No. 6). The sample was prepared from Eastman's No. 83 material, stated to be terpene-free, by repeated treatment with concentrated H_2SO_4 until the acid did not discolor appreciably. The oil was then washed with water and steam-distilled. The absorption bands at 12.3 and 13.85 μ, shown in Curves 3B, were obtained with a 3.4% solution in limonene, after steam-distillation in the trap by the usual procedure.

Effect of Temperature During Storage of Canned Orange Juice (Curves 3C)

The pack of Florida Valencia orange juice, described here, was made on March 31, 1948. In order to get sufficient oil for analytical work from 2-liter samples of juice, procedures were adopted to give a high oil content. The oranges used received no surface treatment after picking, except washing. The juice was simply pasteurized; no deaerating or deoiling procedures were applied. The canned juice was allowed to stand hot for about a minute to sterilize the container, then cooled promptly to 70°F.(21°C.) or below. The samples used for the initial examination were frozen until analyzed as shown in Table 2; the rest of the pack was at room temperature for 7 days until con-


Section
I
II
III
IV
V
μ
2.58
3.41
5.70
5.98
7.78
8.54
8.89
9.23
11.84
12.10
12.35
12.85
13.53
3A
3B
3C
3D
S
0
0
0
1
2
3
4
5
5A
6

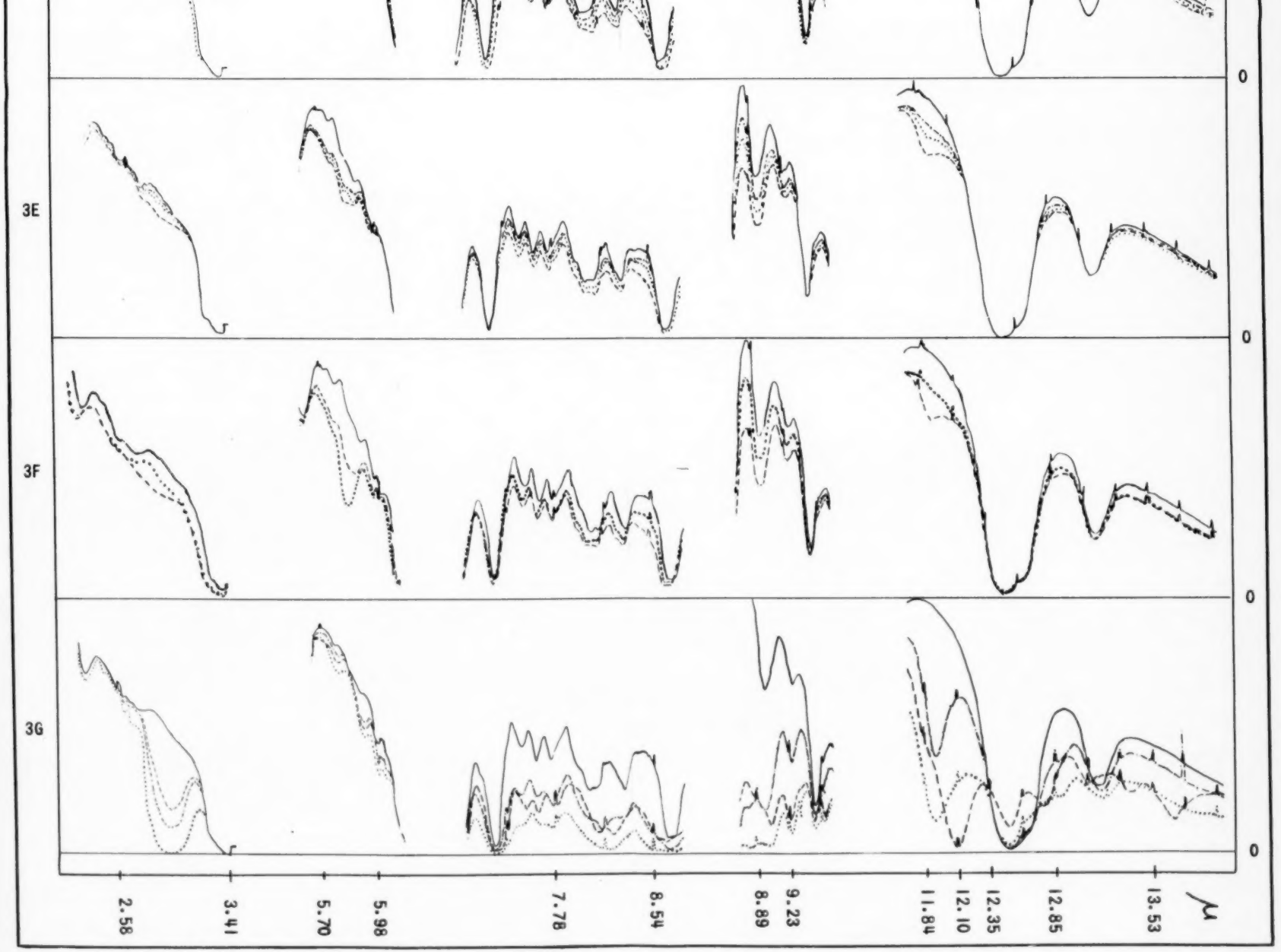


Figure 3. Infrared spectrograms of various oil phases. The curves labelled "S" at the top of the figure represent the "source curve" or "energy curve," applicable as reference for all of the curve-families (3A to 3G). The solid curve in each curve-family represents the absorption of limonene. Curves 3A: . . . . . 6.9% terpineol; _____ 19.6% terpineol, both in limonene. Curves 3B: Band 1, 3.5% caprylic acid, bands 2, 4% 1,4-cineole, bands 3, 3.6% 1,8-cineole, band 4, 3.1% alpha-terpinene, bands 5, 9.0% terpinolene, bands 6, 3.4% p-cymene; all in limonene. Bands 5A represent the absorption of the terpinolene fraction, undiluted. Curves 3C: Oil phases distilled from canned orange juice after 400 days of storage; -.-.- at 45°F ; . . . . . at 70°F.; _____ at 95°F.; all at pH 3.5. Curves 3D: Oil phases distilled from canned orange juice after 4 weeks of storage at 120°F.; -.-.- at pH 4.2; . . . . . at pH 3.5; _____ at pH 2.3. Curves 3E: Oil phases distilled from canned orange juice after 34 weeks of storage at 85°F.; -.-.- at pH 4.2; . . . . . at pH 3.5; _____ at pH 2.3. Curves 3F: Oil phases distilled from phases separated by centrifuging canned orange juice after 315 days of storage at 95°F. . . . . oil from sludge phase; _____ oil from aqueous phase. Curves 3G: Oil phases distilled from canned limonene-citrate systems after 7 days of storage at 175°F.; -.-.- at pH 4.6; . . . . . at pH 3.5; _____ at pH 2.3.

TABLE 2

Effect of temperature of storage

Storage of juice		pH Level during distillation	Observations on distilled oil		
Days	Temperature		Yield	Observed optical rotation, 10 cm. tube	Refractive index, n_D^{20}
	°F.		p. p. m.		
15	Frozen	3.70	382	97.0°	1.4726
16	Frozen	3.70	383	97.0°	1.4726
400	45	3.67	308	96.3°	1.4720
400	70	3.67	319	97.5°	1.4723
400	95	3.68	255	95.9°	1.4727
400	45	5.04	315	97.7°	1.4720
400	70	5.01	270[b]	97.9°	1.4721
400	95	5.13	218[b]	95.5°	1.4727

[b] These 2 values for oil yield are known to be low, because oil droplets were observed clinging to walls of the trap, and these could not be brought up to be measured.

trolled storage at 3 temperatures was instituted. At the expiration of 400 days of such storage, all the samples analyzed were placed at 45°F.(7°C.) until the analyses could be completed, which required 3 days.

The values for optical rotation were obtained by use of a micropolarimeter tube 2 mm. in diameter and a home-made polarimeter consisting of a split-field Polaroid polarizer and a Nicol analyzer; sodium light was used but the temperature during measurement was not controlled. The values are considered to be good to about 0.5°. These results are in agreement with those of other experiments in showing no decrease in rotation at 70°F.(21°C.) and a decrease of only a very few degrees at 95°F.(35°C.), as compared with the values obtained after storage at 45°F.(7°C.).

The decrease in recoverable oil in storage is an outstanding result, always to be observed in similar experiments. It appears from the data of Table 2 that a significant decrease in recoverable oil content took place during prolonged storage even at 45°F.; that the decrease was no greater than this at 70°F.; but that it was significantly greater at 95°F.

The infra-red spectra of the last 3 samples of Table 2, namely the samples of oil distilled from juice after adjusting its pH level to pH 5.0, are reproduced as Curves 3C. Omitting from consideration for the moment Section II of the charts, let us compare Curves 3C with Curves 3A. From the many points of similarity it will be evident that terpineol has been formed during the storage of canned juice. It is also evident from this comparison that the absorption bands of terpineol are so predominant in the spectra of the oil from aged juice that it is very difficult to find evidence of the characteristic bands (Curves 3B) of the products of further ACHD change. Close analysis of the original charts does seem to afford such evidence as a slight "flattening" of the 95°F. curve near 12.1 μ, which may be taken to indicate a trace of alpha-terpinene. But at any rate, the evidence for the formation of terpineol is a good argument for believing that the whole ACHD hypothesis is valid.

The infra-red spectra of the 3 samples obtained by distilling juice of the 3 lots at the natural pH of about 3.7, namely the samples described in the middle section of Table 2, were also obtained, but are not repro-

duced in this paper. In each case the curves differ from the corresponding curves of 3C in showing the presence of slightly more terpineol. However, the differences between the 3 curves themselves are just as great as those of Curves 3C, indicating that the change during distillation at pH 3.7 is very much less than the change during storage of the juice.

The "aging" effects evidenced in Section II of Curves 3C are not to be found in Curves 3A and cannot be ascribed to terpineol. The large absorption at 5.8 μ, increasing with temperature, is ascribable to the carboxylic acid group. Thus an absorption band in this region is manifested by caprylic acid, as shown in Curves 3B. However, all saturated carboxylic acids absorb here, and this particular band does not distinguish between them. It will be observed in Curves 3C that another band, just to the left of the acid band and centering at about 5.75 μ, decreases with temperature of storage. Standard atlases of infra-red spectra *(3)* justify ascribing this band to ester, and Curves 3C, Section II, therefore give evidence of decrease in ester and increase in carboxylic acid content of the distilled oil phase as a function of increase in temperature of storage. The esters that disappear may be largely those which are components of peel-oil, but the acids that appear are only partially derived from these and are partially derived from fruit-pulp constituents, as stated earlier. The alcohols which are formed in the hydrolysis of ester should augment the characteristic alcohol band at 2.9 μ, which is evidenced also by the terpineol produced by ACHD change.

Effect of pH Level During Storage of Canned Orange Juice (Curves 3D, 3E)

The pack of Florida Valencia orange juice used was made on May 26, 1949. One lot of juice was packed at the natural pH level of about pH 3.6; another lot was packed after acidifying to pH 3.1 with hydrochloric acid; while the third lot was raised to pH 4.2 by adding sodium hydroxide. Fairly concentrated solutions of the reagents were used, to avoid unduly diluting the juice. Otherwise the canning procedure was similar to that described in the preceding section. On receipt at this laboratory, the pack was divided for storage at 85°F.(29.5°C.) and 120°F.(49°C.), while a few samples were held frozen. All of the cans of the lot stored for 24 weeks at 120°F. were hard swells when examined, and after this drastic storage it was considered superfluous to raise the pH level before distilling. All of the cans of the other lots retained some vacuum, and in examining them the pH level was raised to about pH 5.0 before distilling, as shown in Table 3.

The infra-red spectra of the oils obtained from the juices stored at the 3 pH levels for 4 weeks at 120°F. are shown in Curves 3D, and those appertaining to the 3 samples stored for 34 weeks at 85°F. are shown in Curves 3E. It will be evident in each case that the lower the pH level of the juice, the greater is the concentration of terpineol in the distilled oil phase. A comparison of Curves 3D and Curves 3E shows that more ACHD change takes place in 4 weeks at 120°F. than in 34 weeks at 85°F., showing again the critical importance of temperature of storage. Curves 3D and 3E are so strikingly similar to Curves 3C, in a qualitative way, as to indicate that raising the pH level of orange juice before canning should have a beneficial effect in inhibiting ACHD reaction during storage, quite analogous to that which would attend upon the maintenance of a lower storage temperature.

That this expectation has a real basis is attested by the results of odor tests made with the 3 oil samples obtained after juice storage of 34 weeks

at 85°F. Observers who were unaware of the identity of the samples stated that the distilled oil from the juice of pH 4.2 was "fragrant," "still-orange-like," "much milder and sweeter," while the oils from both of the juices of lower pH level were characterized as "terpene-like."

The infra-red spectra of the 2 samples of pH level 4.2 held in frozen storage for 4 weeks and for 35 weeks, respectively, and described in Table 3, were also obtained. These spectra are not shown in Curves 3D and 3E, however, because they are so close to those of the samples of pH 4.2 at the higher storage temperatures that they would be hard to distinguish from the latter, when reduced for reproduction.

TABLE 3

Effect of pH level during storage

pH Level		Storage		Observations on distilled oil		
In the can	As distilled	Weeks	Temperature	Yield	Observed optical rotation, 10 cm. tube	Refractive Index, n_D^{20}
			°F.	*p.p.m.*		
3.08	5.00	1	Frozen	263	96.3°	.....
3.58	5.01	1	Frozen	350	96.6°	.....
4.13	5.02	1	Frozen	343	96.6°	.....
3.10	5.10	4	120	177	.....	.....
3.53	4.95	4	120	300	.....	.....
4.26	5.28	4	120	255	.....	.....
4.24	5.15	4	Frozen	316	.....	.....
3.11	5.15	34	85	270	97.0°	1.4736
3.61	5.02	34	85	265	98.3°	1.4732
4.23	5.12	34	85	299	98.0°	1.4678
4.20	5.12	35	Frozen	302	97.6°	1.4731
3.1[c]	3.1[c]	24	120	53	.....[d]	1.4742
3.6[c]	3.6[c]	24	120	187	86.0°	1.4734
4.2[c]	4.2[c]	24	120	313	95.3°	1.4730

[c] Nominal pH values; actual values undetermined.
[d] Insufficient oil obtained to permit determination of optical rotation.

The data of Table 3 lead to certain inferences worthy of discussion. As in the preceding experiment, it appears that considerable alpha-terpineol can be present without causing any appreciable change in refractive index or in optical rotation. The increase of about 1.5° in rotation during 34 weeks at 85°F., as compared with the original frozen samples, should be real and can only be plausibly explained by the disappearance of some natural, optically inactive (or relatively so) natural components from the distilled oil phase. A real decrease in optical activity with decreasing pH level is not encountered except after 24 weeks at 120°F. There appears to be a definite correlation between pH level and yield of

recoverable oil after warm storage; the more acid the juice, the lower the yield. This was certainly to be observed after 24 weeks at 120°F., beyond doubt. Again, there is an apparent slow decrease in yield of distilled oil in frozen storage even at pH 4.2.

Differences Between the Two Phases Obtained by Centrifuging (Curves 3F)

The orange juice pack made on March 31, 1948, which was used as described above for investigating the effect of storage temperature (Curves 3C), was also used in experiments in which the canned juice was centrifuged in 500-ml. bottles at 2,000 r.p.m. for 15 minutes. The radius of rotation was 4 inches at the tops and 9 inches at the bottoms of the bottles. The aqueous upper phase was decanted off and distilled at its natural pH level. The gelatinous lower phase was redispersed in 2 liters of water and distilled therefrom.

After 315 days of storage at 95°F.(35°C.) the upper phase yielded 139 p.p.m. and the lower phase 109 p.p.m. of oil, both in terms of volume of juice. The oil from the upper phase was considerably more disagreeable in odor than the oil from the lower phase, but even the latter was not fragrant. The infra-red spectra of these 2 oil samples are shown in Curves 3F, Figure 3. It will be observed that considerably more terpineol was present in the oil from the upper than in the oil from the lower phase. But the particular point of interest is that there is, on the contrary, considerably more acid in the oil from the lower than in the oil from the upper phase. This is manifested by the absorption band of the carboxylic acid group centering at 5.8 μ (Section II of Curves 3F compared with band No. 1 of Curves 3B). This bespeaks the presence of a considerable amount of water-insoluble acid formed during storage of the juice.

After 314 days of storage at 70°F., the upper phase yielded 153 p.p.m. and the lower phase 152 p.p.m. of oil. After storage at this lower temperature the oil from the lower gelatinous phase seemed to be very fragrant and quite free from objectionable odor components, while the oil from the upper aqueous phase contained all the "stale" odor components which are formed during storage under these conditions. This contrast in odor is very striking and has been confirmed by other experiments. The conclusion is that the "stale" components exist in the can in water solution. The infra-red spectra of these 2 oil samples were also obtained but are not reproduced in this paper, because the total amount of terpineol formed under these storage conditions is not large, and the divergences of the two curves are not great enough to be clearly evident when the curves are reduced for reproduction. However, it is unmistakably apparent on our original charts that, again, there is more acid in the oil from the lower gelatinous phase than in the oil from the aqueous phase, although the terpineol is present almost entirely in the oil from the aqueous phase. In other words, the evidence is qualitatively like that of Curves 3F, but the absorption bands are much less pronounced. This evidence, that a considerable amount of carboxylic acid is present in the fragrant oil from the lower phase after 70°F. storage, shows that this water-insoluble acid is odorless or at least not malodorous. However, smaller amounts of relatively water-soluble acids are shown by the IR charts to be present in the oil from the aqueous phase, both after 70°F. and 95°F. storage.

Direct Evidence of the Formation of Oil-Soluble Acid During "Aging"

Early in this investigation, a pack of Florida Valencia orange juice was made on March 15, 1947, and placed in storage at 45°, 70°, and 95°F.(7°, 21°, and 35°C., respectively). In examining this pack the mistake was made of distilling for 75 minutes at the natural pH level of the juice, thus probably incurring an undue amount of change during the analysis. Furthermore, during the storage of this pack we were not yet ready to determine the IR absorption spectra, or to determine the optical rotation of the distilled oil phase. However, certain data obtained with this pack are presented in Table 4, because they include values which show that the distilled oil phase contains oil-soluble acids in concentrations which depend upon the temperature of storage.

TABLE 4

Formation of oil-soluble acid during storage

	Temperature of storage	Days stored	Juice		Steam-distilled oil		
			pH	Normality	Yield	Refractive index, n_D^{20}	Acid content, microeq. per ml.
	°F.				p. p. m.		
Set 1	45	229	3.51	0.190	350	1.4724	48, 46
	70	228	3.55	0.187	357	1.4722	70, 79
	95	227	3.60	0.190	253	1.4733	103, 126, 134, 131
Set 2	45	237	3.56	0.187	364	1.4726	30, 33, 32
	70	236	3.58	0.187	361	1.4725	50, 51, 52
	95	234	3.55	0.193	327	1.4730	91, 104, 97, 105, 105
Set 3	45	247	3.40	0.187	322	1.4726	48, 46, 56
	70	251	3.52	0.188	350	1.4731	95, 97, 93
	95	248	3.57	0.191	293	1.4729	141, 137, 129

In the course of this work the volume of oil obtained from 1,500 ml. of juice ran as low as 0.45 ml., and the use of any titration method for the determination of oil-soluble acid in the expected small concentration in the oil was not deemed feasible. Recourse was had to a chromatographic method based on the work of Graff and Skau *(9)*. These authors used magnesium oxide columns, stained with phenol red, for the separation of fatty acids. However, we were more interested in the determination of total acid by a band-width method; a project which seemed feasible because of the considerations discussed by Wilson *(31)*.

It was found that the band-widths obtained with magnesium oxide were not only narrow but were markedly affected by the time of passage through the column. A medium which did yield practical results was prepared by evaporating a dilute solution of KOH and phenol red in methanol on fine, well-cleaned glass powder. The medium was frequently stirred during the last stages of drying in an oven. The pH level of an aqueous suspension of the dried powder was found to be about pH 10, indicating that the medium probably actually is a mixture of finely divided $KHCO_3$, K_2CO_3, and phenol red with the glass powder as a diluent. The medium was packed by standard shaking, in a machine, in glass tubes carefully selected to be of 4 mm. inner diameter, with a sintered glass filter at the bottom. The solvent in all cases was a middle fraction of Skelly C, distilled over KOH. The calibrations with purified caprylic acid and the

actual determinations on the distilled orange oil samples were carried out with the same batch of medium. The method is therefore merely an empirical comparison with a standard, rather than one involving true chromatograms. A photograph of a set of tubes used in calibration is shown in Figure 4. It will be observed that the correlation of band-width with acid concentration is not linear but is fairly reproducible. In the actual analyses of the orange oils, a micropolarimeter tube holding the volume of 0.311 ml. was used as a "volumetric flask"; this volume of oil was made up with solvent to 25 ml. and aliquots taken as desired for checking. In every case the columns were "developed" by 3 successive 2-ml. portions of solvent. Dry air at a standard pressure

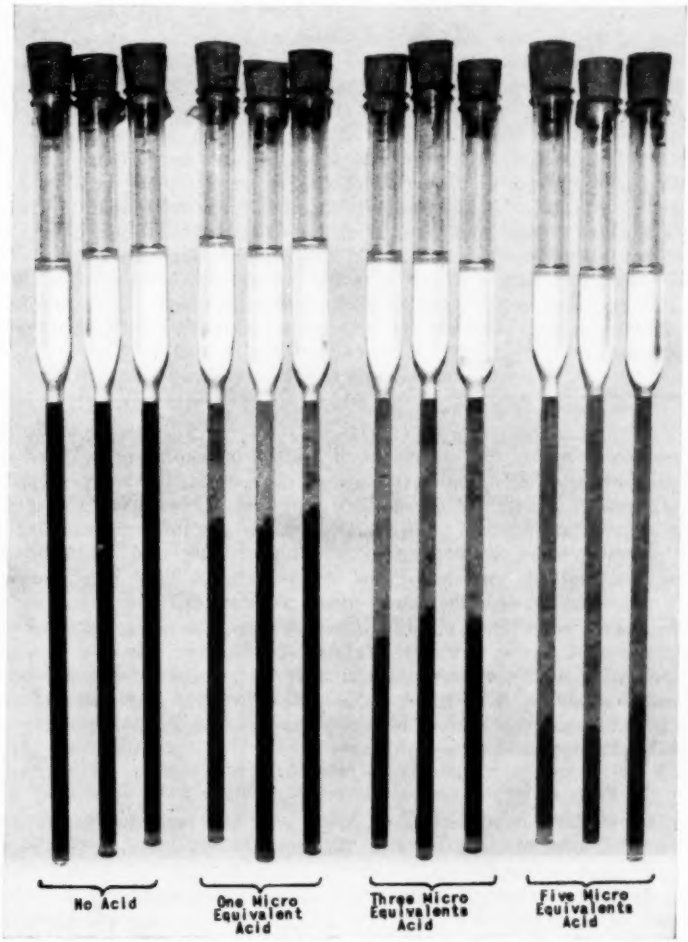


Figure 4. Chromatogram tubes showing the band widths produced by standard samples of caprylic acid.

of 20 p.s.i.g. was applied throughout to filter and to develop the columns in a reasonable length of time. Nevertheless, the method is tedious and time-consuming, and was not applied to our later packs, because it was considered to have served our present purpose in showing by direct evidence that an oil-soluble acid is formed during aging of canned orange juice, to an extent which depends on temperature of storage. This confirms the infra-red evidence obtained with later packs.

All of the chromatogram tubes used in obtaining the oil-soluble acid values of Set 1, Table 4, were filled and packed at the same time, but were emptied and refilled for Set 2 and again for Set 3.

Kesterson and McDuff *(13)* found the ester content of Florida Valencia orange oil not to exceed 1.63%, calculated as acetate of $C_{10}H_{18}O$. If this were completely hydrolyzed and all the resulting acid collected in the distilled oil phase, the concentration of acid would be only about 70 microequivalents per ml. From the data of these authors a more typical ester value would be perhaps one-third of this, or about 25 microequivalents per ml. It is evident, therefore, that a considerable proportion of the oil-soluble acid formed in the aging of canned orange juice must have its origin in fruit-pulp constituents. It is conceivable, of course, that some of the oil-soluble acid found was actually formed by hydrolysis during the 75-minute distillation of the juice in these experiments. Perhaps most of that found after storage at 45°F. might be ascribed to this cause. Even so, it is an inescapable conclusion that the oil-soluble acid is formed during warm storage, and probably by hydrolysis both of peel-oil and of fruit-pulp esters.

Waxy Non-Odorous Acid from Peel-Oil-Free Juice

When peel-oil-free orange juice is distilled in the oil-trap apparatus of Figure 2, a waxy deposit collects at point *O*. The precursor of this substance is necessarily, therefore, a fruit-plup constituent. When distilling orange juice which has a normal oil content, the waxy substance is presumably a solute in the distilled oil phase. The substance is largely soluble in dilute aqueous potassium hydroxide, and infra-red examination has shown it to consist principally of a higher fatty acid. Although the results are merely confirmatory of Matlack's work *(18)* they reassured us further as to why the lower phase after centrifuging aged canned orange juice could yield oil which was fragrant although containing considerable acid, and as to why our values for total oil-soluble acid distillable from aged juice were considerably higher than those which could be attributed solely to the hydrolysis of peel-oil esters. The yield of such waxy acid substance obtainable from peel-oil-free juice is sufficient to justify these considerations.

Packs of Purified Limonene Dispersed in Citrate Buffer Solutions

The need for quantitative exploration of the limonene-citrate buffer system has been outlined early in this paper. Several exploratory experiments have been carried out, but of these only 2 will be described in detail. The first of these (A) describes the results of short storage at a temperature high enough (175°F.[79.5°C.]) to make the presence of both cineoles and of alpha-terpinene unmistakably demonstrable. The second (B) was designed to investigate the possible effect of oxygen on the ACHD reactions.

A. The effect of storage at 175°F.(79.5°C.) for 1 week (Curves 3G, Figure 3). This pack illustrates the drastic effect of storage at 175°F.(79.5°C.) for 1 week, in contrast with storage at 120°F.(49°C.) for 8 days. The great importance of pH level is also demonstrated.

The limonene was a Podbielniak fraction from orange oil, boiling at 66°C., at 18 mm., purified just before use by methanolic KOH, as described above. Its optical rotation was not then measured, but similar samples always have a rotation of 101° or higher. Then 15 ml. was dispersed by a Waring blendor in a solution of 10 g. of "Sure-Jell" pectin in 500 ml. water. The pH level of 2.3 was attained by use of a 1% solution of citric acid monohydrate; the other pH levels by use of potassium citrate buffers at the same total citrate concentration. In making the pack, 25 ml. of the limonene emulsion and approximately 500 ml. of a hot citrate solution were placed in each 307 × 409 can. This means that each can originally contained about 600 mg. of limonene, corresponding to about 1,200 p.p.m. or at least 4 times the concentration that would be encountered in commercially packed orange juice. The reason for this was that a very extensive conversion of the limonene to water-soluble substance was anticipated.

The yields and optical rotations of the distilled oil phases are given in Table 5. It is immediately obvious from the yield data in this table that extensive conversion of limonene to water-soluble substance has taken place under all conditions of storage. A very striking fact is that the yield of recoverable oil after storage at 175°F. was at a minimum at pH 3.5, rather than at pH 2.3 as might have been expected. This is confirmed by the appearance of the reaction systems on opening the cans: the system at pH 3.5 was almost clear while those at pH 4.6 and pH 2.3 were definitely

TABLE 5

Changes in limonene dispersed in citrate buffer

Original pH level	After storage			
	For 7 days at 175°F.		For 8 days at 120°F.	
	Yield, mg. per can	Optical rotation, 10 cm. tube	Yield, mg. per can	Optical rotation, 10 cm. tube
2.30	159	24.2°	375	99.7°
3.52	72	.....*	329	102.1°
4.60	160	83.0°	369	102.2°

* Yield of oil insufficient to fill the micropolarimeter tube.

more turbid and about equal to each other in this respect. The conclusion, which is confirmed by the infra-red evidence, is that hydration to water-soluble substance has reached its maximum at pH 3.5 while at pH 2.3 the ACHD reactions have proceeded further, to a dehydration stage, characterized by the presence of considerable amounts of hydrocarbons other than limonene.

The infra-red spectra of the 3 distilled oil phases obtained after storage at 120°F. were obtained but are not reproduced in this paper. They show the formation of terpineol, to an extent which increases with decreasing pH level, after the manner of the orange juice experiment portrayed in Curves 3D in Figure 3.

The infra-red spectra of the 3 oil phases distilled after storage of the reaction systems at 175°F. are reproduced in Curves 3G. Our interpreta-

tion of the extensive ACHD change at 175°F. which is made obvious by these spectra may be summarized as follows:

At pH 4.6 the distilled oil phase contains something like 30% alpha-terpineol, with relatively minor amounts of products of further ACHD change.

At pH 3.5 the hydration to alpha-terpineol has been still more extensive, so that the terpineol content of the distilled oil phase is estimated to be about 50%. However, the presence of both cineoles is now evident, with the 1,4-cineole predominating over the 1,8-isomer. The total cineole concentration appears to be about 5-7%. A small amount of alpha-terpinene (perhaps 3%) appears to be present.

At pH 2.3 the ACHD change has been so extensive that the limonene content of the distilled oil phase cannot be much more than 25%. The terpineol content (about 20-25%) is also less than it is in either of the other samples. Alpha-terpinene is now unmistakably present as demonstrated by the strong characteristic absorption of 12.10 μ, and by weaker bands at 6.21 and 14.6 μ. Its concentration is estimated to be about 15%. The 2 cineoles appear to be present in about equal concentration, and together amount to about 10-15% of the distilled oil phase. In addition, new and definite absorption bands appear, most noticeably at 12.78, 13.08, 13.38, and 13.67 microns. These we believe to be ascribable, in all probability, to terpinolene and gamma-terpinene, as discussed above. The fact that these bands show up so distinctly means that these substances whose infra-red absorption is relatively weak must be here present in considerable concentration. It is noteworthy that even under the drastic conditions of this experiment there is no evidence of cymene having been formed.

B. Changes in a canned limonene-citrate system, with and without oxygen. The technic adopted for obtaining the oxygen-free lot of this experiment while establishing the presence in each can of a known amount of limonene, made use of the known fact that an acid product during heating in an unenameled can rapidly becomes so highly reduced that a condition of hydrogen overvoltage exists at the acid-metal interface *(14, 17)*.

The apparatus used in packing this lot is sketched in Figure 5. The conical depression *D* was pressed into the can-end before its use. Citrate buffer alone (500 ml. of 0.1 *M.* citrate, pH 3.5) was introduced into each can which was then closed at room temperature under 29 inches of vacuum. The residual oxygen was then consumed by processing the closed cans for 7 hours in boiling water. The cans were continuously rotated axially during th rocess. After cooling to room temperature overnight the glass assembly shown in Figure 5 was firmly clamped (using a frame which is not shown in the figure) down onto rubber gasket *G* set in the countersink of the can end, forming a gas-tight seal. Nitrogen of high purity was passed through the assembly, exiting through delivery funnel *F*, into which the hypodermic syringe *H* had not yet been introduced. After several minutes the syringe, containing 0.50 ml. of purified limonene, was introduced into the funnel, against the effluent nitrogen and just as it made gas-tight contact with rubber sleeve *R*, stopcock *S* was opened to let the nitrogen escape there. The limonene was then allowed to run down into *D*, and the funnel allowed to drain as well as possible. The piercing needle *N* was then screwed down through threaded lug *L* which is affixed to the holding frame. The point of *N* is a phonograph needle. When it pierced the end, the springy pressure of the can end kept the hole sealed until the needle was retracted a little, whereupon the reduced pressure inside the can sucked the limonene quite smoothly and completely into the can. The piercing assembly was then withdrawn from the center neck of the assembly and a soldering copper introduced through it, against effluent nitrogen, to solder-tip the puncture. Nothing had thus been introduced except limonene and nitrogen.

In packing the control or air-containing lot, 500 ml. of the buffer and 0.5 ml. of the limonene were placed in each can which was then closed at room temperature and atmospheric pressure. The cans were size No. 2 (307 × 409), so that the headspace volume in both lots was approximately 80 ml.

In order to give the oxygen of the air-containing lot increased opportunity to exert whatever effect it might have, the cans of both lots were rotated axially at room temper-

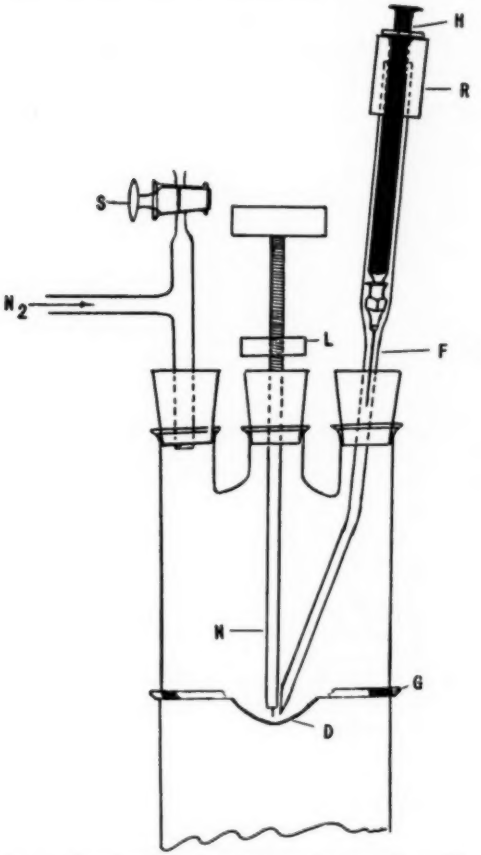


Figure 5. Apparatus for obtaining air-free canned samples.

ature for 18 hours. Following this, the rotation was continued while the cans were given 7-hour boiling-water process in lieu of prolonged warm storage. Such a process probably would not yield the products of ACHD change in the same proportions as those resulting from prolonged storage at a lower temperature, but the experiment should nevertheless legitimately evaluate any effect of oxygen on the course of the ACHD changes.

It will be observed from some of the results presented in Table 6 that the presence of oxygen did not affect the decrease in optical rotation of the oil phase which took place during the process. The presence of oxygen perhaps slightly reduced the yield of recoverable oil, although the differences indicated in the table are scarcely beyond the probable error of the determination. One definite effect of oxygen was that the oil phase distilled from the air-containing samples had a yellow color which was

TABLE 6

Effect of the presence of air on a canned limonene-citrate system

Description of lot	pH during distillation	Yield of oil	Yield of oil	Optical rotation, 10 cm. tube
Immediately after filling:		*mg.*	%	
Air-free	6.60	718 (2 cans)	85.5[f]	102.9°
Air-containing	6.75	754 (2 cans)	90.0[f]	102.0°
After processing:				
Air-free	7.15	559 (2 cans)	77.7[g]	94.9°
Air-containing	12.20[h]	839 (3 cans)	74.1[g]	94.9°
Air-free	5.10	841 (3 cans)	78.1[g]	94.1°
Air-containing	5.10	806 (3 cans)	71.2[g]	94.1°

[f] The percentage yield values for the unprocessed samples, distilled immediately after filling, measure the mechanical losses of limonene during filling, sampling, and distilling.

[g] After processing the amount of limonene has been reduced not only by the mechanical losses as above, but also by ACHD conversion to water-soluble substance. The yield values given after processing are computed as percent of the limonene which had been found to be recoverable before processing; that is, the differences between these values and 100 measures the chemical conversion to water-soluble substance during the processing.

[h] It was intended to distil one pair of samples (air-free and air-containing) at pH 7 and another pair at about pH 5, thinking that a comparison between the two pairs might be valuable in case an acid were formed by action of oxygen. By miscalculation, the sample indicated was treated with a gross excess of KOH. However, since the results of the other pair showed that no perceptible amount of steam-volatile oil-soluble acid was formed, it turned out that this error, in its results, merely confirmed the fact that the products of ACHD change are not reactive toward bases.

almost completely absent from the oil phases distilled from the air-free samples. However, the outstanding result of the experiment was that the infra-red spectrograms of the oil-phases distilled from the air-containing samples were nevertheless almost perfectly congruent with those from the air-free samples, in the case of both pairs referred to above. This was just as true in the carbonyl region (5.8 to 6.0 μ) and in the regions characteristic of cymene (Curves 3B, bands No. 6, Figure 3), as in the other regions of the spectra. The infra-red evidence of change during the heat-process in all cases would be largely accounted for by the formation of about 15% terpineol, and the amounts of the cineoles and of terpinene indicated were so small as to make it necessary to ascribe most of the loss of optical rotation to the postulated presence of terpinolene.

The oil phases distilled from the air-free samples were just as strongly terebinthine in odor as those distilled from the air-containing samples, and when the two oil phases were added to peel-oil-free juice, observers in a blind taste-test found the flavors of the two types of sample to be equally bad and practically indistinguishable.

SUMMARY

The characteristically orange-like flavor of orange juice is due to the presence of a small amount of peel-oil, without which the juice is relatively insipid. The "terebinthine" off-flavor which develops in canned orange juice at temperatures higher than 70°F.(21°C.) is due to some of the substances produced by chemical interaction of the terpenes of the peel-oil with the acid juice. The 2 components, kept separate in nature, are intermingled when the juice is expressed for canning. Evidence as to the nature of the interaction is afforded by infra-red spectrograms of the oil phase obtained from the juice by distillation. The reactions are the reversible

acid-catalyzed hydration-dehydration (ACHD) reactions of the terpenes. The evidence shows the dependence of these reactions upon pH level as well as upon temperature. The constituent oxygen that is present in the alcohols and ethers formed by these reactions owes its presence to the chemical addition of water; not to atmospheric oxidation.

These ACHD reactions are best studied by means of limonene-citrate packs, which yield an oil phase uncomplicated by the presence of the other natural components of orange oil. Although the reactions of limonene in an acid environment are well established qualitatively in the literature of chemistry, little information of a quantitative nature, such as the relative proportions of the various reaction products at equilibrium under various conditions, is available. The exploratory experiments described in the present paper give some idea of the general nature of the system, and indicate that it should be feasible to undertake a more quantitative study.

Experimentation with canned limonene-citrate buffer systems has shown that the ACHD reactions are not affected fundamentally by the oxygen originally enclosed in a can of orange juice, so that the development of terebinthine flavor in warm storage would not be prevented by the complete exclusion of oxygen, even if this could be achieved in the canning process. However, certain minor effects of oxygen, superimposed upon the ACHD changes under special conditions, are described.

The infra-red evidence also attests to the hydrolysis of esters during storage of canned orange juice. This is confirmed by direct evidence of the formation of oil-soluble acids at a rate which depends upon the storage temperature. Much of the oil-soluble acid is an odorless water-insoluble acid of high molecular weight, formed by hydrolysis of fruit-pulp constituents. However, other acids of lower molecular weight and greater solubility in water, derived from both peel-oil and fruit-pulp constituents, are believed to be contributors to a "stale" off-flavor which can be best perceived in canned juice which is never subjected to a storage temperature higher than 70°F. This "stale" flavor, however, is quite complex and involves other reactions, the discussion of which is reserved for a future communication.

Acknowledgments

The writers are indebted to Dr. R. R. McNary of the University of Florida Citrus Experiment Station, and to Dr. O. R. Alexander of this laboratory, for suggesting the applicability of infra-red spectrophotometry in the investigation of the distilled oil phase, and to the Food Technology Department of the Massachusetts Institute of Technology for enlisting the aid of Spectrophotometric Laboratory of the Institute in a preliminary check of this possibility.

LITERATURE CITED

1. Austerweil, M. G. An isomer of cineole (1,4 cineole). *Bull. soc. chim. France*, **45**, 862 (1929).
2. Barnard, D., Bateman, S., Harding, A. J., Koch, N. P., Sheppard, N., and Sutherland, G. B. B. M. An infra-red spectroscopic investigation of double-bond structure in simple acyclic terpenes and derivatives thereof. *J. Chem. Soc.*, **1950**, 915 (1950).
3. Barnes, R. Bowling, Gore, Robert C., Liddel, Urner, and Williams, Van Zandt. *Infrared Spectroscopy*. 1944. Reinhold Publishing Corporation, New York.
4. Bartholomew, E. T., and Sinclair, Walton B. Apparatus for the determination of volatile citrus oils. *J. Assoc. Offic. Agr. Chemists*, **28**, 339 (1945).
5. Boyd, J. M., and Peterson, G. T. Quality of canned orange juice. *Ind. Eng. Chem.*, **37**, 370 (1945).
6. Curl, A. Laurence, and Veldhuis, M. K. The origin of the off-flavor which develops in processed orange juice. *Fruit Products J.*, **26**, 329 (1947).

7. FRANK, ROBERT L., AND BERRY, ROBERT E. The isomerization of 8 (9) p-menthene. *J. Am. Chem. Soc.*, **72**, 2985 (1950).
8. GASCOIGNE, R. M. The detection and estimation of alpha terpinene by means of the diene synthesis. *J. Proc. Roy Soc. N. S. Wales*, **74**, 353 (1940).
9. GRAFF, MORRIS M., AND SKAU, EVALD L. Colored chromatograms with higher fatty acids. *Ind. Eng. Chem., Anal. Ed.*, **15**, 340 (1943).
10. GUENTHER, ERNEST, AND ALTHAUSEN, DARRELL. *The Essential Oils*. Vol. II, 1949, D. Van Nostrand Co., New York.
11. HENRY, R. E., AND CLIFCORN, L. E. Deterioration in flavor of canned orange juice. *Canning Trade*, **70** (31), 7, 22 (1948).
12. KEFFORD, J. F., MCKENZIE, H. A., AND THOMPSON, P. C. O. The effect of oxygen on flavour deterioration and loss of ascorbic acid in canned orange juice. *Food Preservation Quarterly*, **10**, 44 (1950).
13. KESTERSON, J. W., AND MCDUFF, O. R. Florida citrus oils, commercial production methods and properties of essentials oils. Univ. Florida Agr. Expt. Sta., *Bull.*, **452** (1948).
14. KOHMAN, E. F., AND SANBORN, N. H. Factors affecting the relative potentials of tin and iron. *Ind. Eng. Chem.*, **20**, 1373 (1928).
15. KUNZ, LUDWIG. U. S. Patent 1,735,118 (November 12, 1929).
16. LEWIS, V. M., AND MCKENZIE, H. A. Amperometric determination of dissolved oxygen in orange juice. *Anal. Chem.*, **19**, 643 (1947).
17. LUECK, R. H., AND BLAIR, H. T. Corrosion in the tin can. The electrochemical relations of iron and tin. *Trans. Electrochem. Soc.*, **54**, 257 (1928).
18. MATLACK, M. B. The fatty constituents of California Valencia orange pulp. *J. Org. Chem.*, **5**, 504 (1950).
19. NELSON, E. K., MOTTERN, H. H., AND EDDY, C. W. Nitrogenous constituents of Florida Valencia orange juice. *Fruit Products J.*, **12**, 231, 250 (1933).
20. NOLTE, ARTHUR J., AND VON LOESECKE, HARRY W. Chemical and physical characteristics of the petroleum ether soluble material of fresh and canned Florida Valencia orange juice. *Food Research* **5**, 457 (1940).
21. POWER, F. B., AND CHESNUT, V. K. The odorous constituents of peaches. *J. Am. Chem. Soc.*, **43**, 1725 (1921).
22. POWER, F. B., AND CHESNUT, V. K. The odorous constituents of apples. II. Evidence of the presence of geraniol. *J. Am. Chem. Soc.*, **44**, 2938 (1922).
23. RIESTER, D. W., BRAUN, O. G., AND PEARCE, W. E. Why canned citrus juices deteriorate in storage. *Food Indus.*, **17**, 742, 850 (1945).
24. SIMONSEN, J. L., AND OWEN, L. N. *The Terpenes*. 2nd Ed., Vol. I, 1947, Cambridge University Press.
25. SMITH, ARTHUR H. A protein in the edible portion of orange. *J. Biol. Chem.*, **63**, 71 (1925).
26. WAGNER, GEORG. On camphor glycols and a 4-hydroxy alcohol from limonene. *Ber.* **23**, 2307 (1890).
27. WALLACH, O. Information on the terpenes and essential oils. Part I. The crystalline terpineols. *Ann.*, **275**, 103 (1893).
28. WALLACH, O. Information on the terpenes and the essential oils. Paper No. 26. *Ann.*, **277**, 105 (1893).
29. WHITE, JONATHAN W., JR. Composition of a volatile fraction of apples. *Food Research*, **15**, 68 (1950).
30. WILL, R. J. Some phases of the citrus by-product industry in California. *J. Ind. Eng. Chem.* **8**, 78 (1916).
31. WILSON, J. NORTON. A theory of chromatography. *J. Am. Chem. Soc.*, **62**, 1583 (1940).

RETENTION OF NUTRIENTS IN COMMERCIALLY PREPARED SOYBEAN CURD[a]

CAREY D. MILLER, HELEN DENNING,[b] AND ADELIA BAUER[c]
Foods and Nutrition Department, University of Hawaii
Agricultural Experiment Station, Honolulu, Hawaii

(Manuscript received September 15, 1951)

Soybean curd or "tofu" continues to be a favorite food of the people of Japanese ancestry in Hawaii. In 1950 a study of the consumption of Japanese foods, by families of 85 university students of Japanese ancestry who were attending home economics classes, showed that 96% of the families still use tofu; 60% of them use it often (daily to 3 times per week), and 36% use it occasionally (1 to 4 times per month).[d]

The Board of Health reported to the senior author that on July 31, 1951, 26 companies or persons on the Island of Oahu (City and County of Honolulu, civilian population 315,000) were licensed to produce tofu commercially. During August we determined that one small operator had discontinued business. By inquiry of the other 25 producers, the amount of tofu prepared each day in the week was determined. Most of the factories make tofu 6 days a week; 3 operate 7 days; and 1 or 2 only 3 or 4 days a week. Some of the "factories" are of the small, home type. There was great variation in the output, the smallest producing only 20 lb. daily and the largest more than 1000 lb. daily. From the weekly output of each factory, the total daily average production of Japanese type bean curd on the Island of Oahu was calculated to be about 5000 lb. Only one licensed factory now produces Chinese type bean curd several times a week with an average daily production of 85 lb.

When soybean curd is made from soybeans, a product without crude fiber and with protein of high digestibility is obtained *(7)*. According to work done elsewhere *(2, 3, 7)* soybean protein has a relatively high biological value. Increased costs for both labor and dried soybeans in Hawaii have raised the price for a pound of tofu from 5 cents (prewar) to 15 cents at the present time. Even at this price it continues to be a relatively economical source of a highly digestible protein.

A previous study in our laboratory *(4)* indicated a low retention of thiamine (18%) when soybeans were made into soybean curd by the usual commercial process for making Japanese tofu. The thiamine was determined by the rat growth method on samples from one small tofu factory.

The present study was designed to determine the thiamine retention by chemical methods, to extend the study to other nutrients, and to obtain products from more than one factory.

---

[a] Published with the approval of the Director as Technical Paper No. 240, Hawaii Agricultural Experiment Station.

[b] Present address: Pennsylvania State College, State College, Pa.

[c] Present address: National Dairies Research Laboratory, Oakdale, Long Island, N. Y.

[d] Stella Okita, a senior student in the Home Economics Department, made this study.

SAMPLES AND METHODS

Three factories cooperated by furnishing the samples required. The average daily tofu production of factory No. 1 was 200 lb.; of factory No. 2, 1400 lb.; and of factory No. 3, 350 lb.

Details of the methods of preparing Japanese type of bean curd in Hawaii vary somewhat for each factory, but the essential points in the manufacturing process are as follows: Dried green or yellow soybeans are washed and soaked in tap water for 6 to 8 hours. The soaked beans are crushed mechanically, usually with some type of stone grinder, with the addition of about 3 times the volume of water during the grinding.

The liquid mass containing the crushed beans is then brought to the boiling point. The quantity of the mixture heated, and the period and manner of heating, vary in each factory and are established largely on the basis of equipment and experience. The hot mixture is strained and pressed in bags made of strong coarse cloth. The residue remaining after pressing is called "kirazu," the strained liquid is soybean milk or "tonyu."

The protein in the tonyu is precipitated as a flocculent curd by the addition of a solution of calcium chloride. Here again, the manner of adding the solution of calcium chloride, the way in which the mixture is stirred, and the time elapsing before the curd is molded vary with each processor and are based on his experience in producing a satisfactory product. The mixture is poured into a wooden frame lined with cloth, the liquid is drained off, and the curd is subjected to pressure for 20 to 30 minutes. When the curd is well set, it is cut into approximately 3-inch cubes, each weighing about 1 lb. The blocks of tofu are placed in a tub or can of fresh water at room temperature for the wholesale or retail trade.

Tofu is made fresh daily, usually 6 times a week. The homemaker keeps the tofu in water with or without refrigeration until it is used. It is normally used the day it is purchased if the tofu is eaten with shoyu (soybean sauce) or added to various dishes. If it is to be kept overnight, most homemakers cook it by immersion in boiling water or by frying.

It was impossible to get accurate data on the quantity of the different products obtained from a specified weight of dried soybeans. From the rough estimates made by the operators, approximate yields were calculated. These figures were used in estimating the retentions of nutrients in products from all 3 factories (Tables 2 and 3). Since tofu is made by rule of thumb methods and exact weights are not used, the addition of a little more water, a little longer heating period, a little more pressure on the curd, could all influence the quantity of nutrients in the samples analyzed.

Samples obtained from each factory were: 1, 1 lb. of dried soybeans; 2, 2 lb. of kirazu; 3, 2 quarts of tonyu; 4, 2 quarts of water pressed from the precipitated tonyu; and 5, 3 blocks of tofu. The blocks of tofu were left in a beaker from 1½ to 2 hours, and the water which separated from the blocks was drained off and analyzed. About 290 ml. of water separated from 3 blocks of tofu in that length of time. All 5 products were obtained from one factory on the same day.

Proximate composition, calcium, phosphorus, iron, thiamine, riboflavin, and niacin were determined on dried soybeans and the following products made from the same lot of beans: soybean milk, soybean curd residue, and soybean curd. The liquid pressed from the curd at the factory and that which exuded at the laboratory were also analyzed for the three vitamins. Retention of vitamins was determined for products obtained from all 3 factories and retention of protein, calcium, phosphorus, and iron from 2 factories.

Thiamine was determined by the thiochrome method, riboflavin by the fluorometric method, and niacin by the microbiological method *(1)*. For all other nutrients, the methods, essentially those recommended by A.O.A.C., have been described in detail elsewhere *(5)*.

RESULTS AND DISCUSSION

Figures for proximate composition, calcium, phosphorus, and iron of soybeans and products are given in Table 1. The varietal names of the soybeans (imported from mainland U.S.A.) used by each factory were unknown but they were all yellow or pale green in color. Some variation in composition is to be expected when a number of samples of unknown

TABLE 1

Proximate composition, calcium, phosphorus, and iron contents of soybeans and soybean products

Food	Moisture	Protein	Fat	Crude fiber	Total reducing sugars	Carbohydrate (by difference)	Ash	Calcium	Phosphorus	Iron
	%	%	%	%	%	%	%	*mg. per 100 g.*	*mg. per 100 g.*	*mg. per 100 g.*
Soybeans, dried										
Sample 1*	9.4	34.9	18.6	4.5	8.5	28.1	4.5	208	640	7.2
Sample 2	8.2	37.0	17.9	3.9	10.2	28.6	4.4	196	632	7.9
Sample 3	10.0	36.2	17.7	4.0	10.3	28.4	3.7	156	656	8.2
Soybean residue										
Sample 2	75.3	8.9	3.6	3.1	1.1	8.2	0.9	107	173	2.0
Sample 3	81.4	5.0	1.4	2.4	0.5	9.2	0.6	53	101	1.3
Soybean "milk"										
Sample 2	91.0	4.1	1.7	0	0.7	2.7	0.5	(39)	77	1.0
Sample 3	93.3	2.7	1.3	0	0.7	2.3	0.4	13	75	0.6
Soybean curd										
Sample 2	83.2	11.5	4.2	0	1.0	0.2	0.9	186	149	1.7
Sample 3	83.8	9.4	4.2	0	0.6	1.9	0.7	111	175	1.5

*Sample numbers correspond to factory numbers.

TABLE 2

Calcium, phosphorus, and iron in dried soybeans and their retention in soybean products

Food	Approximate yield of product per kg. beans	Protein			Calcium			Phosphorus			Iron		
		In 100 g. or ml.	Total	Retention	In 100 g. or ml.	Total	Retention	In 100 g. or ml.	Total	Retention	In 100 g. or ml.	Total	Retention
		g.	*g.*	%	*mg.*	*mg.*	%	*mg.*	*mg.*	%	*mg.*	*mg.*	%
Soybeans, dried													
Sample 2	1.0 kg.	37.0	370	100	196	1960	100	632	6320	100	7.9	79	100
Sample 3	1.0 kg.	36.2	362	100	156	1560	100	656	6560	100	8.2	82	100
Soybean residue													
Sample 2	1.0 kg.	8.9	89	24	107	1070	55	173	1730	27	2.0	20	25
Sample 3	1.0 kg.	5.0	50	14	53	530	34	101	1010	15	1.3	13	16
Soybean "milk"													
Sample 2	8.3 liters	4.1	340	92	(39)	(3237)	(165)	77	6391	101	1.0	83	105
Sample 3	8.3 liters	2.7	224	62	13	1079	69	75	6225	95	0.6	50	61
Soybean curd													
Sample 2	2.7 kg.	11.5	310	84	186	5022	157†	149	4023	64	1.7	46	58
Sample 3	2.7 kg.	9.4	254	70	111	2997	192†	175	4725	72	1.5	40	49

†Increase in calcium content the result of using calcium chloride as a precipitant.

variety and origin are used. Dry soybean Sample 2 was from a recent shipment which had a lower moisture content than the other 2 samples.

The protein contents of the soybean milk and soybean curd from Factory 2 were much higher than those from Factory 3. It is the belief of the operator of Factory No. 2 that more soybean milk and more tofu can be obtained from freshly harvested dried soybeans than from those which have been stored for a long period. The protein content of tofu Sample 2 (11.5%) is almost 20% greater than that of Sample 3 (9.4%). Previous results from our laboratory *(6)* showed tofu to have 9% protein and tofu made and analyzed in Japan has been reported to contain 6.5% protein *(8)*.

In order to determine the fate of the original nutrients, the calculations shown in Tables 2 and 3 were made. Table 2 includes the approximate retention of protein, calcium, phosphorus, and iron in the three products, soybean residue, soybean milk, and soybean curd. The seeming lack of consistency in the retentions is probably the result of several factors, namely—inaccurate estimates of the quantity of the products obtained from a given weight of soybeans, the difficulties of collecting a truly representative sample of the entire lot being handled in one day, and different methods of processing, as well as differences that would be expected to occur as a result of natural variation in lots of soybeans.

Kirazu is now rarely used for human food in Hawaii but is usually sold to pig producers. Although kirazu retains all the crude fiber, compared with tofu, it contains somewhat less protein, fat, and calcium; approximately the same amount of phosphorus, iron, and niacin; and more thiamine and riboflavin. It should constitute a valuable stock feed.

The retention of the various nutrients by tofu is of greatest interest as that is the product normally used for human food. From the data presented in Table 2, it may be concluded that tofu retains approximately 70 to 80% of the protein, 60 to 70% of the phosphorus, and 50 to 60% of the iron. However, the possibility exists that some of the iron found in the finished product may be the result of contamination from iron equipment in the factory. The apparent high retention of calcium in the tofu results from the use of calcium chloride as a precipitant for the protein.

From Table 3 it is obvious that the quantities of the three B vitamins extracted in the milk are greater than are retained in the residue, as might be expected, since they are water-soluble. Although the calculated retentions are not uniform for the three products (kirazu, tonyu, and tofu) from the three factories, these inconsistencies are again probably the result of inaccurate estimates of the yields of the different products, the difficulties of sampling, and other factors discussed above.

The results in Table 3 demonstrate clearly that not only is it impossible to extract all of the B vitamins from the soybean residue, but that when the milk is made into tofu, large losses of the B vitamins occur. The water pressed from the soybean curd contains more of the vitamins than the tofu itself, and as the blocks of tofu stand, the water which oozes from them contains the vitamins, thus further reducing the amounts in the tofu. The blocks of tofu were allowed to stand in beakers in the laboratory for

TABLE 3

Thiamine, riboflavin, and niacin in dried soybeans and their retention in soybean products

Item	Approximate yield of product per kg. beans	Moisture	Thiamine			Riboflavin			Niacin		
			Quantity		Retention	Quantity		Retention	Quantity		Retention
			In 100 g. or ml.	Total		In 100 g. or ml.	Total		In 100 g. or ml.	Total	
		%	γ	γ	%	γ	γ	%	*mg.*	*mg.*	%
Soybeans, dried											
Sample 1	1.0 kg.	9.4	955	9550	100	273	2730	100	1.83	18.30	100
Sample 2	1.0 kg.	8.2	1720	17200	100	266	2660	100	1.31	13.10	100
Sample 3	1.0 kg.	10.0	853	8530	100	220	2200	100	2.21	22.10	100
Soybean residue											
Sample 1	1.0 kg.	85.3	108	1080	11	25	250	9	0.15	1.50	8
Sample 2	1.0 kg.	75.3	92	920	5	25	250	9	0.25	2.50	19
Sample 3	1.0 kg.	81.4	101	1010	12	21	210	10	0.18	1.80	8
Soybean "milk"											
Sample 1	8.3 liters	94.6	67	5560	58	30	2490	91	0.14	11.60	63
Sample 2	8.3 liters	91.0	123	10210	59	30	2490	94	0.30	24.90	(190)
Sample 3	8.3 liters	93.3	96	7970	93	23	1910	87	0.21	17.40	79
Water pressed from precipitated soybean "milk"											
Sample 1	5.5 liters	.....	70	3850	40	32	1760	64	0.15	8.25	45
Sample 2	5.5 liters	.....	129	7095	41	51	2805	105	0.32	17.60	(134)
Sample 3	5.5 liters	.....	90	4950	58	29	1595	72	0.20	11.00	50
Soybean curd											
Sample 1	2.7 kg.	85.2	59	1590	17	15	405	15	0.12	3.24	18
Sample 2	2.7 kg.	83.2	81	2190	13	27	730	27	0.23	6.21	47
Sample 3	2.7 kg.	83.8	78	2100	25	17	460	21	0.17	4.59	21
Water drained from soybean curd upon standing											
Sample 1	0.5 liters	.....	53	265	3	11	55	2	0.09	0.45	2
Sample 2	0.5 liters	.....	80	400	2	13	65	2	0.16	0.80	6
Sample 3	0.5 liters	.....	45	225	3	10	50	2	0.11	0.55	2

about 2 hours prior to sampling, but when sold in the stores, the blocks are kept in containers of water, so that considerable leaching must continue.

Although the fresh shipment of dried soybeans (Sample 2) had a higher initial thiamine content than either of the other two samples, the tofu produced from it is only slightly superior in thiamine to Sample 3. The water pressed from the tofu which goes down the sewer and the water drained from the tofu which is discarded both showed higher values for Sample 2 than for the other two samples.

Although the retentions can be considered only approximate for reasons previously discussed, they may be summarized by stating that soybean milk may retain 50 to 90% of the thiamine, approximately 90% of the riboflavin, and 60 to 80% of the niacin found in the dried soybeans from which the milk was made. On the same basis soybean curd or tofu may be expected to retain from 10 to 25% of the thiamine, 15 to 25% of the riboflavin, and 20 to 40% of the niacin. If the tofu is kept in water and if it is allowed to stand and a portion of the water drains out, the three B vitamins will decrease because the vitamins tend to pass from the tofu into the water.

Because the thiamine and riboflavin values for 100 g. of tofu for Samples 1 and 2 showed wide variations, samples of tofu were obtained from these two factories a year after the first assays were made. The values for thiamine were almost identical with those shown in Table 3 (Sample 1, 59 γ per 100 g.; Sample 2, 82 γ per 100 g.). Riboflavin for both Samples 1 and 2 were the same (18 γ per 100 g.) which means that the value for Sample 2 was much lower than when originally determined but was more in line with the values for the other two factories.

It is believed that the figures for protein, calcium, phosphorus, iron, thiamine, riboflavin, and niacin for Japanese soybean curd given in Tables 2 and 3 reliably represent the composition of this food as found on the Honolulu market.

SUMMARY AND CONCLUSIONS

Proximate composition and calcium, phosphorus, and iron have been determined for samples of dried soybeans used by 3 factories, and for samples of soybean curd residue (kirazu), soybean milk (tonyu), and soybean curd (tofu) from 2 factories making Japanese-type soybean curd.

Thiamine, riboflavin, and niacin were determined for dried soybeans, kirazu, tonyu, tofu, and the water pressed from tofu from 3 factories.

The soybean residue (kirazu) retains enough of all the nutrients to constitute a valuable stock feed. It is useful as a human food only to a limited extent because of its high crude fiber content.

Exact data on the yields of the different products from a definite weight of dried soybeans could not be obtained, but on the basis of the best estimates that could be made, Japanese soybean curd retains approximately 70 to 80% of the protein, 60 to 70% of the phosphorus, 50 to 60% of the iron, 10 to 25% of the thiamine, 15 to 25% of the riboflavin, and 20 to 40% of the niacin of the beans from which the curd is produced. An apparent high retention of calcium (157 and 192%) results from the use of calcium chloride as a precipitant for the protein.

Approximately half of the water-soluble vitamins are lost in the water pressed from the curd.

Soybean curd of the Japanese type contains approximately 10% of a high quality vegetable protein with no crude fiber, and when precipitated with calcium chloride is (per unit of weight) as good a source of calcium as milk.

In view of the world shortage of good quality proteins, the production and use of soybean curd should be encouraged in all parts of the world where animal proteins are in short supply and the protein and calcium of the diets are low.

Acknowledgments

The authors gratefully acknowledge the cooperation of the Aloha Tofu Factory, Kanai Nissei Shokuwai, and Yokoyama Tofu Factory; the Board of Health; and all operators who supplied information on tofu production.

LITERATURE CITED

1. Association of Vitamin Chemists, Inc. *Methods of Vitamin Assay.* 1947, Interscience Publishers, Inc., New York.
2. Cahill, W. M., Shroeder, L. J., and Smith, A. H. Digestibility and biological value of soybean protein in whole soybeans, soybean flour, and soybean milk. *J. Nutrition*, **28**, 209 (1944).
3. Chang, I. C. L., and Murray, H. C. Biological value of the protein and the mineral, vitamin, and amino acid content of soymilk and curd. *Cereal Chem.*, **26**, 297 (1949).
4. Miller, C. D. Thiamin content of Japanese soybean products. *J. Am. Dietet. Assoc.*, **21**, 430 (1945).
5. Miller, C. D., Ross, W., and Louis, L. Hawaiian-grown vegetables: proximate composition; calcium, phosphorus, total iron, available iron, and oxalate content. Univ. Hawaii Agr. Exp. Sta., *Tech. Bull.*, **5** (March 1947).
6. Miller, C. D. Japanese foods commonly used in Hawaii. Hawaii Agr. Exp. Sta., *Bull.* **68** (1933).
7. Pian, J. H. C. Biological value of the proteins of mung bean, peanut and bean curd. *Chinese J. Physiol.*, **4**, 431 (1930).
8. Saiki, T., Higuchi, T., Kondo, M., and Matsuzawa, K. *The Chemical Analysis of Food in Japan.* 1931, The Nankodo, Tokio, Kioto, Japan (In Japanese).

A STUDY OF THE CONTROL OF THE SANITARY QUALITY OF CUSTARD-FILLED BAKERY PRODUCTS IN A LARGE CITY[a]

ABRAHAM E. ABRAHAMSON, RUBIN FIELD, LEON BUCHBINDER, AND ANNA V. CATELLI

Bureaus of Food and Drugs and Laboratories, Department of Health, New York, N. Y.

(Manuscript received October 8, 1951)

In the manufacture and distribution of baked goods, the widening gap between producer and consumer has introduced many problems relative to handling of ingredients by more individuals, and to preparation, storage, refrigeration, distribution, and merchandising activities. The factor of time lapse between production and consumption also adds to these problems.

It has long been recognized that baked goods designated as custard-filled pastries may become a health hazard unless they are prepared in a sanitary manner and controlled. The fillings furnish the moisture, nutrients, and physical state favorable for the growth of microorganisms normally occurring in the ingredients, or which may gain entrance by careless handling, and some of these, under unfavorable conditions, may be responsible for causing illness, particularly food infection or food poisoning.

This latter problem has engaged the interest of workers in the Department of Health of New York City for a number of years, because of the repeated occurrences of outbreaks. This paper deals with the experience of a group of investigators in attempting to control production of these products.

The overall public health problem presented by these products on the state and national levels also is clear, as is demonstrated by Coughlin and Johnson *(6)* and U. S. Public Health Service Reports *(9)*.

Many workers have indicated the magnitude of this problem and have described their efforts at its control. Gilbert, Coleman, and Laviano *(10)* recovered large numbers of staphylococci from the filling of cream puffs and eclairs involved in a food poisoning outbreak. Catheart, Ryberg, and Merz *(3)* failed in an attempt to sterilize custard filling with ultra violet rays of 2500-2950 A, but found that by lowering the pH and using formulas containing pure fruit fillings, the growth of *Staphylococcus aureus* and *Salmonella enteritidis* was inhibited, the latter to a greater extent *(4)*. They also stated that in order to have a marked effect on the growth of *Staphylococcus aureus*, the amount of lemon juice and grated lemon rind required for a standard custard is so great that a commercially unacceptable flavor results.

Catheart, Godkin, and Barnett *(2)* reported that when the pH of a cheese cake filling was below 5.12, the growth of *Staphylococcus aureus* was retarded. When lactic acid was substituted for citric acid in cheese cake

[a] Read at a Laboratory Section Meeting of the American Public Health Association Convention, at the 78th Annual Meeting in St. Louis, Mo., October 31, 1950.

filling, it produced a better flavor and checked the growth of *Staphylococcus aureus* at pH 4.42 and 4.67.

Stritar, Dack, and Jungewalter *(15)* suggested that reheating of custard-filled puffs and eclairs at 190.6° to 218.3°C. for 30 minutes would destroy staphylococci without impairing the flavor or appearance of the product. They cautioned that at higher temperatures excessive browning occurs unless egg whites are substituted for milk in the manufacture of the shell. Korff *(12)* reported in 1936 that 15 bakeries in Baltimore which were reheating custard-filled pastries found the method to be satisfactory. Gilcreas and Coleman *(11)* advised rebaking of eclairs, chocolate cream pies, and Boston cream pies for 20 minutes at 216° to 220°C. This neither damaged their appearance nor affected their palatability. New York City bakers who tried the reheating process found these products unacceptable to their trade *(1)* because the filling became rubbery and shrank.

Cathcart, Ryberg, and Merz *(5)* found that "merely bringing custards to a second boil after the addition of the thickening mix, rendered them free of both *Staphylococcus aureus* and *Salmonella enteritidis* with which they had been inoculated;" and that "baked custard pies, which had been inoculated with *Salmonella enteritidis* and *Staphylococcus aureus* before baking," were free of these two organisms as they left the oven.

Segalove and Dack *(13)* recommended that foods be stored at refrigerator temperatures (4.0° to 6.7°C.) since staphylococci do not produce enterotoxin when held at such temperatures for as long as 4 weeks. Dack *(7)* stated that several factors lead to the natural growth of staphylococci in food. One, however, the holding of food at room temperature, which thus permits growth, could be readily controlled by refrigeration.

Topley and Wilson *(16)* used the following emphatic language in urging prophylactic steps: "The protection of custard and cream fillings and made-up meat dishes, from staphylococcus infection of human origin requires the greatest care and vigilance. In heavy nasal carriers of *Staphylococcus aureus*, the hands are often infected and there may be suppurative skin lesions on the hands and arms. Measures little short of a surgical aseptic technique may have to be taken to prevent infection of the ingredients and stringent regulations should be laid down about the adequate heating and subsequent refrigeration of food."

PLAN OF STUDY

In 1936 it was decided that a survey be made to determine the bacteriological status as well as methods of production of commercially prepared custard-filled baked goods, such as napoleons, eclairs, cocoanut custard pies, and nesselrode pies. It was hoped that it would be possible on the basis of survey findings to introduce appropriate control measures.

Description, Method of Manufacture, and Comment on Some of the Custard-Filled Bakery Products Studied

Eclairs. Eclairs are filled with a preparation containing essentially water, sugar, whole milk powder, cornstarch, whole egg, salt, possibly citric acid and vanilla, and chocolate or other flavor. These ingredients are cooked to a plastic consistency. The mass is then allowed to cool after which it is pumped by means of a narrow gauge

nozzle into a pre-baked sausage shaped shell about 4 or 5 inches in length. The shell is streaked, after filling, with a chocolate or other flavored icing.

Napoleons. Napoleons consist of alternating layers of a thin, flaky, prebaked pastry dough and layers of custard prepared essentially in the same manner as described under eclairs. The top layer of dough contains a vanilla flavored icing which is frequently streaked with a chocolate icing for decorative purposes. The layers of custard are applied by a broad-bladed icing spatula to large layers of pastry. After layers of dough and custard filling and icing are applied, the large cake is cut up into smaller sections which constitute individual servings.

Cocoanut custard pie. The pre-formed dish-like shell is filled with a fluid custard containing water, sugar, powdered whole milk, salt, eggs, cornstarch, and flavoring. A sprinkling of shredded cocoanut is applied either before or after the fluid is discharged into the shell just prior to baking. Cocoanut custard pies have enjoyed a privileged position from a food poisoning viewpoint. The assumption has been that they are above suspicion bacteriologically. It was decided to include custard-filled pies in this study because the filling is similar to that used in other custard-filled products, and is an excellent medium for bacterial growth.

Nesselrode pie. Nesselrode pie, unlike cocoanut custard pie, does not receive a final heat treatment. A preformed dish-like shell is filled with a preparation containing light cream, egg yolks, sugar, fruit, pure chocolate, rum flavor, vanilla, and topped with heavy whipped cream. The preparation is mixed in 3 stages: 1, if imitation cream is used for topping, it is homogenized, heated, then cooled and aged for 48 hours; if heavy cream is used it also is aged; 2, light cream or milk, egg yolks, and sugar are mixed and heated to 160° to 170°F.(71° to 76°C.); and 3, fruit, flavor, or chocolate is mixed with freshly prepared gelatin. Mixtures 2 and 3 are incorporated and applied by ladle or spatula to the prebaked shell and refrigerated for several hours. The whipped topping (Mixture 1) or whipped aged cream is applied before shipping.

Nesselrode pie is a new baker's product which was relatively recently introduced on a large commercial scale and is rapidly becoming popular as a rich dessert. This product can be considered as belonging to the group of custard-filled baked goods. There was no information available concerning the bacteriological status of nesselrode pies at the time of this investigation.

Methods

Samples of the several products studied were obtained from eating establishments, retail stores, and directly from the wholesale source. Specific information in the following categories were sought: bacterial count, coliform organism count, *Staphylococcus aureus*[b] count, enterococcus count, and hydrogen ion concentration of the custard; time lapse since delivery of the product to the seller; temperature at which the product was held at sampling time, and method of processing to control sanitary quality.

Sampling methods. Samples of various custard-filled baked goods were collected from 5 or 6 different eating places or bakeries one day a week between the hours of 9 A.M. and 1 P.M. They were handled during sampling in the same manner in which they were served to the public. In sampling napoleons and eclairs, 6 individual portions were taken. Cocoanut custard and nesselrode pies were secured whole and uncut in original pie plates. All products were delivered to the laboratory under conditions (temperature and time lag) which would simulate that which would have prevailed during the serving and consumption of these products in the restaurant.

Bacteriological methods. The custard products were tested quantitatively on the day received in the laboratory for the presence of staphylococci of the food poisoning type, enterococci, coliform organisms, and total bacteria. The pH of each sample was also determined. Portions of the custard of all 6 eclairs or napoleons and several parts of the cocoanut custard and nesselrode pies (enough to make 11 g.) were each weighed and placed in a sterile electric mixer (Oster type) together with 99 ml. of sterile water. The mixture was agitated for 2 minutes. Additional decimal dilutions were made and

[b] *Micrococcus pyogenes* var. *aureus*.

inoculated into pour plates of several selective media for the various isolations as follows:

Isolation	Agar medium	Decimal dilutions
Staphylococcus aureus	Chapman's	1/10 to 1,000,000
Enterococci	Sodium azide	1/10 to 1,000,000
All bacteria	Tryptone glucose extract milk	1/10 to 1,000,000
Coliform bacteria	Sodium desoxycholate	1/10 to 1,000

One ml. of sterile skim milk was added to each plate that was to receive sodium desoxycholate agar.

The technique for the examination of plates for staphylococci and enterococci, and their further identification and enumeration was described by Dangler and Steffen *(8)*. Estimation of the total count of all bacteria and of the number of coliform organisms were performed according to procedures described in Standard Methods for the Examination of Dairy Products, 9th Edition, 1948. Arbitrary standards of bacterial quality were set up for the purpose of the study. The estimated plate count was not to exceed 100,000 bacteria per g. and the coliform count 10 per g.

A preliminary study was made in 1946 and 1947 of the bacteriological status of custard-filled pastries. A number of samples of eclairs, napoleons, and cocoanut custard pies were examined. In this study, it was found that the first two products, more often than not, yielded counts greater than the standards indicated above. An attempt was made to control the flora of these two products by use of citric acid or citrus juices. It was found that although the counts did become satisfactory with this procedure, the taste of such pastries was not completely acceptable to the consumer. The preliminary study of cocoanut custard pie revealed, surprisingly, that 9 of 14 samples had excessively high counts. Corrective measures in sanitation and more thorough heat treatments improved this product.

A Study of Custard-Filled Pastries Manufactured in 65 Bakeries

Following this preliminary study, it was decided to continue the bacteriological survey of custard-filled pastries. Through March, 1950, a total of 420 samples of eclairs, napoleons, cocoanut custard pies, and nesselrode pies were collected from sources supplied by 65 wholesale bakeries.

At the outset, satisfactory production in 18 bakeries was indicated by bacterial criteria, while the opposite was observed for the other 47 bakeries (Table 1). The processes of manufacture and method of distribution of the 47 bakeries which produced unsatisfactory custard baked goods were

TABLE 1

Bacterial counts of custard-filled products from 18 bakeries originally classified as satisfactory and from 47 bakeries originally classified as unsatisfactory

Bakery classification	Number of samples with total colonies per g.			
	Under 100,000	100,000-999,000	Over 1,000,000	Total
Satisfactory	64	3	0	67
Unsatisfactory	38	26	51	115
	Number of samples with coliform organisms per g.			
	Negative[b]	Positive	Total	
Satisfactory	60	7	67	
Unsatisfactory	27	88	115	

[b] Negative, less than 10 per g.; positive, 10 or more per g.

then reviewed. This study disclosed one or more conditions to be responsible for bacterial contamination in each case:

1. Cooking was generally ineffective because of insufficiency in time or temperature.
2. Cooling was carelessly performed, permitting contamination during long exposure in the bake shop or after transfer to a vessel which may not have been properly cleaned.
3. Transfer of the cooled custard filling to eclair shells, pastry or pie shells was carelessly done permitting contamination by unclean fillers, vessels, spatulas, and frequently by needless personal contact.
4. Possibilities of contamination of the cooked custard were present at each subsequent stage of handling.
5. A complete lack or disregard of the knowledge of sanitary procedure was evident.
6. Equipment was of a kind which did not lend itself to easy cleaning and sterilization. It was found that custard filling was frequently transferred to empty tin cans which had been used for packing fruit or liquid eggs, and which were not entirely clean.
7. When produced at the wholesale level, the time lag from completion of cooking to delivery to restaurants varied from 6 to 14 hours and frequently more.
8. Few distributors had equipment for continuous refrigeration during transit in warm weather.
9. While manufacture of most custard-filled baked goods is discontinued during warm weather, most producers do not consider the danger of exposure of their products from before noon to after dinner (11 A.M. to 8 P.M.) to room temperature of the restaurant or bakery salesroom (70° to 80°F.) during the remainder of the year.

Recommendations in Methods and Equipment

Methods. In the case of eclairs, in order to minimize the possible opportunities for contamination of the custard filling by human handling, recommendation was made for cooking to at least 200°F.(93.5°C.) for 10 minutes under steam pressure, with agitation to prevent scorching, and then cooling in the same vessel. The cooled custard was then to be transferred by sanitary pump and lines to a sanitary filler.

Though the trade practice in the production of napoleons is to apply the custard filling by clean spatula, it was recommended that the properly cooked and cooled custard filling should be applied by a series of fish tail shaped nozzle tips on a filler manifold.

For cocoanut custard pie it was recommended that: 1, fundamental sanitary procedure be followed in the preparation of the custard mix which was lacking in many bakeries at the outset of the study; 2, the quantity of mix made should be limited to assure short holding periods; and 3, the baking process should be adequate to completely and thoroughly heat the pie filling. Exposure in the oven of the average commercial pie to about 375°F.(190.5°C.) for 20 minutes is adequate.

For nesselrode pie it was recommended that only pasteurized cream or a pasteurized shortening mixture, freshly cracked high quality eggs, and freshly prepared gelatin be used in the making of this product. This was necessary because these pies were unsatisfactory principally due to the fact that the mixture of vegetable shortening, butter, milk or cream was homogenized without heat treatment (pasteurization) and stored under refrigeration for 24 to 48 hours for aging before whipping. When aged pasteurized cream was used instead of the mixture, the bacteriological condition of these products was greatly improved. Similar improvement was noted when the vegetable shortening mixture was heated to a temperature equivalent to pasteurization and then homogenized. The use of freshly cracked eggs, instead of frozen eggs which had been permitted to thaw for a considerable length of time before use, and the use of freshly prepared gelatin were additional factors which improved the sanitary quality of this pastry.

Equipment. All equipment used in these operations should be of such construction as to facilitate cleaning and sterilization. The cleaning procedure should be undertaken immediately after use of the equipment and the equipment be sterilized immediately before use. The equipment recommended was to achieve heating, cooling, and transferring of custard filling from the original vessel to the pastry shell without exposing

the filling to contamination. This recommendation for the mechanical handling of custard filling was carried out with some variations as follows:

1. The more usual method consisted of the use of a steam-jacketed kettle in which the custard filling was cooked under constant agitation. The kettle was designed for use with a coolant. Thus, the custard filling was cooked and cooled in the same vessel. The custard so made was then pumped by sanitary pump and lines to a sanitary filler.

2. An especially designed 3-cylinder horizontal continuous heat transfer unit was constructed *(14)*. This permitted the pre-mixed custard-filling ingredients in the form of a slurry to be subjected to one cylinder to heating at 230°F.(110°C.) for 15 seconds; to cooling in the second cylinder by city water supplied at an operating rate of 25 gallons a minute, and to chilling to 40°F.(4.5°C.) in the third cylinder by direct expansion refrigeration. The chilled custard filling was transferred by sanitary pump and lines to a sanitary filler. This method has produced custard-filled eclairs and custard filling of consistently excellent sanitary quality.

3. Another device was designed for continuous cooling. This was installed between a steam-jacketed kettle with agitators and a sanitary filler. The device was a horizontal jacketed tube cooled by brine refrigeration. The product was first cooked in the kettles and pumped into this tube. The custard was agitated by a rotating spiral. The custard emerged at a temperature of 40° to 50°F.(4.5° to 10°C.) and was discharged to a sanitary filler. This arrangement involved no personal handling of the mix. The cooling device, however, was difficult to clean. Occasional samples from this plant were unacceptable bacteriologically probably because of contamination in the cooler after good cooking procedure.

Of the group of 47 bakery operators, 17 attempted by various means to improve the sanitary quality of their custard-filled products; 6 introduced 3 improvements, 2 introduced 2 improvements, and 1 introduced 1 improvement. The measures taken were:

Engaged bacteriologist	8
Improved sanitation	8
Improved processing	8
Installed mechanical handling devices	5
Instituted pH control	2

In the remaining 30 bakeries no changes were made which would result in improvement.

A new series of samples of the several custard products were obtained from these 47 bakeries thereafter. Significant differences are indicated in Table 2 in bacteriological samples taken from the 17 bakeries which at-

TABLE 2

Bacterial counts of custard-filled products from 17 bakeries which made improvements in their operation and 30 bakeries which did not

Bakery status	Samples with total counts per g.							
	Under 100,000		100,000-999,000		Over 1,000,000		Total	
	No.	%	No.	%	No.	%	No.	%
Improved	96	71	19	14	21	15	136	100
Unimproved	42	42	30	29	30	29	102	100
	Samples with coliform organisms per g.							
	Negative[c]		Positive		Total			
	No.	%	No.	%	No.	%		
Improved	86	63	50	37	136	100		
Unimproved	27	26	75	74	102	100		

[c] See footnote [b], Table 1.

tempted to improve their custard-filled baked goods, as compared with those from the remaining 30 bakeries which did not. Statistical analysis of the bacteriological results of the examination of baked goods produced by the 2 groups before steps were taken to improve practices, revealed no such differences. It is noted that the percentage of satisfactory samples as indicated by total counts less than 100,000 is much greater in the improved group. The difference in coliform content is even more significant; the percentage of satisfactory samples in the improved group is about 2½ times that in the unimproved group.

Thus it may be concluded that careless and indifferent processing of custard-filled baked goods may result in poor sanitary quality as measured bacteriologically and that it is possible, with reasonable care, to produce pastries of a satisfactory bacteriological quality. The findings in the 30 bakeries thus became an enforcement problem for the Health Department.

COMPARATIVE ANALYSIS OF ALL TYPES OF CUSTARD-FILLED PRODUCTS

In Table 3 all the bacteriological data previously presented have been pooled and reexamined to obtain some idea of the comparative bacteriological status of each product. Comparative analysis of the bacteriological findings for eclairs and napoleons indicates a relatively better sanitary condition in the manufacture of napoleons. It should be noted that 47%

TABLE 3

Summary of findings with each type of custard-filled pastry studied

Type of pastry	Samples with total colonies per g.								Samples with coliform organisms per g.					
	Under 100,000		100,000-999,000		Over 1,000,000		Total		Negative[d]		Positive		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Eclairs	92	52	37	22	46	26	175	100	55	31	120	69	175	100
Napoleons	53	61	15	17	19	22	87	100	41	47	46	53	87	100
Cocoanut custard pie	75	71	19	18	12	11	106	100	97	92	9	8	106	100
Nesselrode pie	20	38	7	14	25	48	52	100	7	14	45	86	52	100

[d] See footnote [b], Table 1.

of napoleon samples were relatively free of coliform contamination whereas only 31% of eclairs were. This is a statistically significant difference. It is also seen that 61% of napoleons had less than 100,000 colonies per g., whereas in the case of eclairs this condition was true in only 52% of the samples. This despite the fact that custard in eclairs is protected from the air while in napoleons it is not. The relatively small number of samples available for comparison may be responsible for this lack of significance. It should be borne in mind that in the manufacture of napoleons, the custard filling is applied by spatula whereas in eclairs, it is applied by machine. It may be concluded from the coliform findings that inasmuch as the fillings in both products are essentially the same, the unsatisfactory bacteriological results obtained in the instance of eclairs is caused by contamination contributed by unclean filling machines.

It is interesting to note that the preliminary study seemed to have had some lasting effects upon the sanitary quality of cocoanut custard

pies since the completed study disclosed 92% of the samples to be relatively free of coliform organisms and 71% to have a total plate count less than 100,000 colonies per g. It is also to be noted that a number of the pies were not refrigerated when taken as samples. There was no evidence of any attempt at the control of the pH of the product.

At first a total of 22 samples of nesselrode pies were taken from 11 bakeries. All but 1 was of unsatisfactory sanitary quality. The procedures of the 10 producers of unsatisfactory pies were reviewed and recommendations were made for changes in technique. Immediately 4 bakeries discontinued manufacture of this product. Then 30 resamples were taken from the 6 remaining bakeries. The products of only 3 bakeries were found to be satisfactory. Two of the remainder also discontinued baking nesselrode pie and the other bakery was installing new equipment at the time of this report.

FINDINGS ON STAPHYLOCOCCUS AUREUS OF THE FOOD POISONING TYPE AND ENTEROCOCCI

Staphylococcus was found in 30% (10 of 33) of eclairs, in 12% (3 of 25) of napoleons, in 3% (1 of 31) of cocoanut custard pies, and in 30% (6 of 31) of nesselrode pies. Enterococci was found in 27% (9 of 33) of eclairs, 24% (6 of 25) of napoleons, 13% (4 of 31) of cocoanut custard pie, and 50% (16 of 32) nesselrode pie. It should be noted that Staphylococcus counts in nesselrode pies were as high as 700,000 per g. and that enterococci counts were as high as 12,000,000 per g.

The problem of sanitary control of custard baked goods has been studied by several workers. A number of methods for sanitary control are available as follows: 1, aseptic handling (sanitary methods and equipment); 2, constant refrigeration of the finished product; 3, adjustment of pH; and 4, reheating or rebaking of the finished bakery product.

The baking industry in New York City has found that pH control and reheating or rebaking are least desirable, since these latter processes do not enhance the acceptability of these baked goods by the consumer. It is our belief that the keystone of control rests in proper sanitary technique, and that refrigeration is a secondary consideration.

DISCUSSION

The present study was made to determine the bacteriological status of custard products and the methods used for their manufacture and distribution in New York City. More than 400 samples of eclairs, napoleons, cocoanut custard pies, and nesselrode pies were obtained from 65 bakeries. These products were studied for the total number of all bacteria present and for the coliform count. Determination of the presence, and number of staphylococci of the food poisoning type and enterococci were also made on a number of samples. The study revealed, on the whole, that the sanitary status of these products left much to be desired. Many bakeries produced custard baked goods whose bacterial content far exceeded any reasonable standard.

Recommendations were made to these bakeries for the proper processing of custard-filled baked products. These consisted essentially of minimizing

personal handling by means of cooking and cooling in the same vessel and by direct mechanical conveyance of the custard to the filling operation. Devices used to accomplish this are described. It was also recommended that sanitary laboratory control of the product be routinely carried out by the bakery.

Some bakers complied with the recommendations and a sanitary product usually resulted. Others, a greater number, did not comply and their products usually remained unsatisfactory. A number of these latter producers were thereupon persuaded to discontinue manufacture of the particular custard-filled product.

The study indicated that a standard of not more than 100,000 total count of all bacteria and 10 coliform bacteria per g. is reasonable and can be met easily by a bakery using proper technique.

It was found, unlike what might have been expected, that napoleons yielded satisfactory samples more frequently than did eclairs. The filling of eclairs is a machine process, whereas the addition of custard in the manufacture of napoleons is carried out by a hand operated spatula. It is possible that the use of unclean filling machines may account for this unexpected finding.

Contrary to expectations, cocoanut custard pie which is a heat treated product was found to be not above suspicion bacteriologically. This condition was easily rectified, however, upon adoption of a stated time and temperature for baking.

The sanitary control of nesselrode pie, a rellatively new product, offered a challenge. The first survey of this product revealed only 1 of 22 samples to be of proper sanitary quality. The product of only 3 of 11 establishments was found to be satisfactory after recommendations for improvement of technique had been made. The remaining bakeries discontinued manufacture pending installation of suitable equipment.

CONCLUSION

This study of commerciallly manufactured custard-filled bakery products of various types revealed that:

1. Unsatisfactory sanitary quality, as measured bacteriologically, usually results when the manufacture of such products is not adequately supervised by a health agency.
2. When recommended sanitary changes in methods and equipment were followed, products were satisfactory.
3. Estimated total plate counts not greater than 100,000 per g. and coliform counts of not more than 10 per g. seem to adequately serve, at present, as bacteriological indicators of sanitary quality of these products.

Acknowledgment

Acknowledgment is made of the invaluable guidance and assistance rendered by Mr. George Kerchner of this department in the analysis of the data.

LITERATURE CITED

1. ABRAHAMSON, A. E. Wholesale Food Division, N. Y. City Department of Health, Bureau of Food and Drugs, Unpublished findings, 1942.
2. CATHCART, W. H., GODKIN, W. J., AND BARNETT, G. Growth of *Staphylococcus aureus* in various pastry fillings. *Food Research*, **12**, 142 (1947).

3. CATHCART, WILLIAM H., RYBERG, RICHARD E., AND MERZ, ALBERT. Staphylococcus and Salmonella control in foods. I. Effect of ultra-violet radiation and ozone in bakery custard and cream fillings. *Food Research*, **7**, 1 (1942).
4. CATHCART, WILLIAM H., AND RYBERG, RICHARD E. Staphylococcus and Salmonella control in foods. II. Effect of pure fruit fillings. *Food Research*, **7**, 10 (1942).
5. CATHCART, WILLIAM H., RYBERG, RICHARD E., AND MERZ, ALBERT. Staphylococcus and Salmonella control in foods. III. Effect of cooking bakery custards. *Food Research*, **7**, 100 (1942).
6. COUGHLIN, F. E., AND JOHNSON, BASCOM. Gastro-enteritis outbreaks from custard-filled pastry. *Am. J. Pub. Health*, **31**, 245 (1941).
7. DACK, G. M. *Food Poisoning*. Revised, 1949, University of Chicago Press, Chicago, Ill.
8. DANGLER, GERTRUDE, AND STEFFEN, GUSTAVE I. Studies of Enterococcal food poisonings. *J. Milk Technol.*, **11**, 242 (1948).
9. Division of Sanitation, Milk and Food Branch. Disease outbreaks conveyed through foods other than milk and milk products in the United States in 1948, as reported by state and territorial health authorities. Federal Security Agency, U. S. Public Health Service, Washington, D. C., 1948.
10. GILBERT, RUTH, COLEMAN, MARION B., AND LAVIANO, ALICE B. Food poisoning due to toxic substances formed by strains of the Cloacae-Aerogenes group. *Am. J. Pub. Health*, **22**, 721 (1932).
11. GILCREAS, F. S., AND COLEMAN, M. B. Studies of rebaking cream-filled pastries. *Am. J. Pub. Health*, **31**, 956 (1941).
12. KOEFF, F. A., *Baltimore Health News*, **13**, 144 (1936, 1937).
13. SEGALOVE, MILTON, AND DACK, G. M. Relation of time and temperature to growth and Enterotoxin production of Staphylococci. *Food Research*, **6**, 127 (1941).
14. SIMON, PHILIP N., PRINCE, RICHARD, AND TMERAK, LOUIS J. Continuous closed line assures purity in eclairs. *Food Ind.*, **27**, 880 (1949).
15. STRITAR, JOSEPH, DACK, G. M., AND JUNGEWAELTER, FRANK G. The control of Staphylococci in custard-filled puffs and eclairs. *Food Research*, **1**, 237 (1936).
16. TOPLEY, G. S., AND WILSON, A. A. *Principles of Bacteriology and Immunity*. 3rd Ed., 1946, Williams and Wilkins, Baltimore, Md.

A STUDY OF THE EFFECT OF VARIOUS SUBSTANCES ON THE VOLATILITY OF DIACETYL AT AN ELEVATED TEMPERATURE[a]

ANNE CONRAD STOLL
College of Home Economics, Drexel Institute of Technology, Philadelphia, Pennsylvania

(Manuscript received October 22, 1951)

The use of diacetyl in the baking industry presents a problem not encountered in other fields. The volatile compound, boiling point 88°C., must be subjected to high temperatures during the baking period. Its effective flavoring action, therefore, depends upon prevention of loss by volatilization. For this purpose, various fixatives and carriers have been suggested and used with diacetyl, but actual comparisons of their efficiency in reducing volatility have not been made. The present paper reports data on the effect of various substances on the volatility of diacetyl at an elevated temperature.

In conducting this work, the laboratory procedure is based on experimentation with liquid dispersions of diacetyl and the selected fixatives. This medium has been chosen in preference to a complex flour mixture in order to simplify the analysis and to minimize indeterminate errors. Since this particular study deals only with the retention of diacetyl in simple liquid mixtures, the results may not be directly applicable to baking processes.

EXPERIMENTAL PROCEDURE

Fixatives. Liquid dispersions were prepared by the dilution of 4.798-g. (5 ml.) calibrated samples of diacetyl to 1 liter. Fixatives were thoroughly combined with the diacetyl before dilution. Then 10-ml. aliquot samples of each dispersion were used for analysis.

The selection of fixatives was made on the basis of their actual and possible application to this work, with consideration of availability, convenience of use, and nontoxicity to the body when consumed in small quantities. The experimental work included the use of glycerol; glyceryl monoacetate, diacetate, and triacetate; propylene glycol; gum arabic, tragacanth, and guaiac; and cottonseed oil. Further tests were conducted with some of the newer emulsifiers and stabilizers: dehydrated gelose extract of Irish Moss; water-soluble cellulose ether; polyoxyethylene sorbitan monolaurate; polyoxyethylene sorbitan monostearate; and polyoxyethylene sorbitan monooleate. Experimentation with aqueous diacetyl solution was also included.

For the preparation of the glycerol, monoacetin, diacetin, and propylene glycol dispersions, 25% of the total volume was added as fixative. The fixative was mixed thoroughly with the diacetyl, then water was added to bring the solution to required volume. The triacetin dispersion was made in a similar manner, but because of its limited solubility in water, a 6% concentration was used.

For the preparation of the gum and sorbitan derivative dispersions, weighed amounts of the substances were wetted with diacetyl and water was added gradually. Here 1 g.

[a]This paper is condensed from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Home Economics at Drexel Institute of Technology, June, 1951.

of gum arabic, gum tragacanth, and the sorbitan derivatives was used, but only 0.5 g. of gum guaiac was used.

The Irish Moss extract dispersion was made by adding 250 ml. of hot water to 1 g. of the powder. After agitation and cooling, the diacetyl and remaining water were added.

Preparation of the cellulose ether necessitated a preliminary wetting of the powder in 250 ml. of water at 90°C. It was then allowed to soak for 30 minutes with occasional stirring. After cooling, the diacetyl was added and then the remaining water was added as ice water.

The combination of the diacetyl and cottonseed oil was a flavor-in-oil dispersion wherein the oil was both the diluent and the fixative.

Temperature and length of heating. The temperature and the length of the heating period used in this experiment were derived from a study of temperatures attained in baking products by Barackman and Bell (1). In their experiments, internal temperatures of various batters and doughs were measured with thermo-couples during the baking process. The maximum temperature attained within most products during baking was found to be 100°C.

For this study, a 15-minute period of heating in an oven[b] regulated at 140°C. was found to produce a time-rate of heating ratio for liquid dispersions that approximated that of baked products. Under these conditions, maximum temperatures ranging from 96-100°C. were attained in blank samples from every series of tests. The temperature of the liquid was recorded from a standard thermometer inserted through the oven into the accessory sample. These readings were considered applicable to the test samples as well.

Calculation of sample weight of diacetyl. In order to calculate the percentage retention of diacetyl after heating, the available weight of diacetyl in aliquot samples had to be determined. The weight of 0.05 ml. in each aliquot portion was computed from the weight of diacetyl delivered by a standard 5-ml. volumetric pipette. The 5-ml. sample was measured into a glass weighing bottle that was immediately stoppered and weighed. The average of 10 weighings was 4.750 g. The same 5-ml. calibrated pipette was used throughout the experiment as was a 10-ml. pipette for sample measurement. Every sample, therefore, was assumed to contain 47.5 mg. of diacetyl prior to heating and gravimetric analysis.

Since the presence of fixatives interferes with the precipitation of diacetyl, all samples were distilled before analyzing. Therefore, to account for errors introduced by the effects of dilution, distillation, and precipitation, samples of each dispersion were subjected to a test analysis that omitted the heating period. Because the results of these tests were consistent within prescribed limits, they were averaged to give the corrected sample weight of diacetyl. The average weight of 47 samples was 44.6 mg. with a standard error of $\pm$ 0.1 mg. This figure was used in the calculation of diacetyl retention in every sample.

Analysis. Three 10-ml. aliquot portions of the freshly prepared diacetyl dispersion were transferred to 125-ml. Erlenmeyer flasks. A fourth 10-ml. portion was placed in a sample vial to allow complete immersion of the bulb of the recording thermometer during heating. The samples were placed in the oven held at 140° $\pm$ 1°C. and heated for 15 minutes. The samples were removed and analyzed simultaneously for diacetyl, using a modification of the method developed by Schmalfuss and Rethorn *(2)*.

After the addition of 20 ml. of water, the samples were distilled at 100°C. directly from the 125-ml. Erlenmeyer flasks in a closed system until distillation ceased. The distillate was collected in a 250-ml. Erlenmeyer flask containing 50 ml. of water, 2 ml. of 20% hydroxylamine hydrochloride, and 3 ml. of 10% nickel chloride held at 0°C. The collection flask was removed and 12 ml. of cold sodium acetate solution was added. The mixture was digested in a boiling water bath for 1 hour, then cooled in ice for 1 hour. The nickel dimethylglyoxime precipitate was filtered off using a tared sintered glass crucible of medium porosity. The precipitate was washed with three 10-ml. portions of ice water and dried at 110-120°C. for 1 hour. In this manner, 6 samples of each dispersion were analyzed.

[b] Weber Electric Drying Oven, Type B.

RESULTS AND CONCLUSIONS

The results of this investigation are summarized in Table 1. It may be concluded that the heat volatilization of diacetyl from liquid dispersions is retarded in varying degrees by the presence of selected fixatives. Of the substances used, the cellulose ether and cottonseed oil were found to be the most effective. This may be attributed to the fact that the cellulose ether gelled on heating, and that the cottonseed oil was used both as the diluent and fixative. A practical study of this type does not admit to theoretical conclusions.

TABLE 1

Average percentage retention of diacetyl after heating in liquid dispersions of fixatives at an oven temperature of 140°C. for 15 minutes

Fixative in dispersion	Average retention	Standard error
	%	%
Aqueous solution	0.0	.....
Glycerol	12.4	±1.0
Polyoxyethylene sorbitan monostearate	13.5	±0.6
Diacetin	14.8	±0.5
Triacetin	14.8	±0.4
Gum guaiac	15.2	±0.6
Polyoxyethylene sorbitan monolaurate	16.3	±0.6
Polyoxyethylene sorbitan monooleate	16.7	±0.8
Gum arabic	18.3	±1.0
Monoacetin	18.4	±0.3
Dehydrated gelose extract of Irish Moss	19.8	±0.8
Propylene glycol	20.3	±0.9
Gum tragacanth	20.5	±1.0
Water soluble cellulose ether[c]	44.8	±0.9
Cottonseed oil	48.3	±0.8

[c] Gelled on heating.

SUMMARY

In this study, samples of an aqueous solution and 14 dispersions of diacetyl and fixatives were heated at an oven temperature of 140°C. for 15 minutes and analyzed for diacetyl retention. The results, expressed as average percentage retention, ranged from 0.0 to 48.3%, with a maximum standard error of ± 1.0%. There was no recovery of diacetyl after heating aqueous solution samples. The majority of fixatives retained from 12.4 to 20.5% of the diacetyl, while the cellulose ether and cottonseed oil retained 44.8% and 48.3%, respectively.

LITERATURE CITED

1. Barackman, R. A., and Bell, R. N. Temperatures attained in baking products. *Cereal Chem.*, **15**, 841 (1938).

2. Schmalfuss, H., and Rethorn, H. Bestimmung des Diacetyls. *Z. Untersuch. Lebensm.*, **70**, 233 (1935).

ACID-TOLERANT BACTERIA IN CITRUS JUICES[a]

L. W. FAVILLE[b] AND E. C. HILL[c]
Florida Agricultural Experiment Station, Lake Alfred, Florida

(Manuscript received October 26, 1951)

Available literature pertaining to the types of bacteria indigenous to citrus juices is very limited. Faville, Hill, and Parish *(1)* reported that bacteria comprised the majority of organisms isolated from samples of frozen concentrated orange juice and that *Leuconostoc* was the predominant genus present, while reports on the occurrence of coliform organisms by Patrick *(6)*, Wolford and Berry *(11, 12)*, and Wolford *(10)* constitute the remaining publications on this subject.

Although the relatively high citric acid content of citrus juices exerts a bacteriostatic effect on most species of bacteria, experience resulting from 5 years of commercial production of frozen concentrated citrus juices indicates that from the standpoint of spoilage of the product, bacteria are probably of significance equal to or greater than normally-occurring yeasts and molds. Hayes *(3)*, for example, has isolated strains of *Lactobacillus brevis* and *Lactobacillus plantarum* which he believes were the causative organisms in several instances where spoilage had occurred in the lower-Brix stages of multiple-stage evaporators during low-temperature vacuum concentration of orange juice. Teunisson and Hall *(8)*, on the other hand, studied organisms isolated from the surfaces of processing equipment in citrus canneries and concluded that "bacteria are probably not a major factor in influencing the quality of orange juice." Conflicting reports such as these have prompted this study of acid-tolerant bacteria in citrus juices.

EXPERIMENTAL PROCEDURE

Source of organisms. Most organisms included in this study have been isolated from experimental lots of orange juice, both single-strength and partially concentrated, which have shown evidence of microbial activity. During the course of 2 seasons many such experimental lots of juice have been packed and stored at different temperatures ranging from −8°F.(−22.2°C.) to 40°F.(4.5°C.). Certain of these packs, particularly those stored at the higher temperatures, have developed "swells." On microscopic examination these swells have been demonstrated to be caused by bacterial rather than yeast activity, as might be expected.

The remainder of the organisms studied were isolated from oranges partially or completely rotted internally. Some of these fruit showed very little or no external evidence of decomposition. The Hamlin oranges shown in Figure 1 illustrate a bacterial rot of this type. Although the rotted fruit (right) cannot be easily distinguished from a sound fruit (left) except for a brown spot located at the stylar end (bottom right), the interior portion of the fruit (upper right) has almost completely deteriorated. In this particular fruit the decomposition was most pronounced in the central cylinder and extended throughout the vesicles to the albedo.

---

[a]Cooperative research by the Florida Citrus Experiment Station and the Florida Citrus Commission, Lake Alfred, Florida. Florida Agricultural Experiment Station Journal Series, No. 15.

[b]Assistant Bacteriologist, Florida Citrus Experiment Station, Lake Alfred, Florida.

[c]Research Fellow, Florida Citrus Commission.

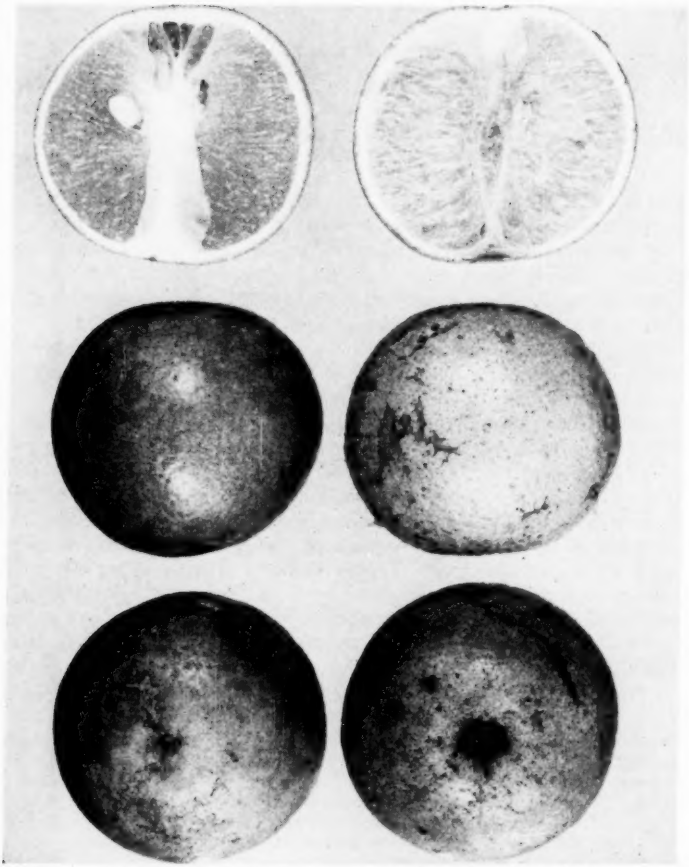


Figure 1. Bacterial rot of Hamlin oranges showing rotten fruit (right) compared to sound orange (left). The only external symptom to denote the presence of rot is the small brown spot located at the stylar end of the fruit (bottom right).

Of 204 bacterial cultures isolated by using Difco dextrose agar as a plating medium, 138 cultures were selected for further study on the basis of their ability to grow in a sterile orange serum at pH 4.0. This serum was prepared by putting orange juice having the desired pH first through filter paper with the aid of Filter-cel and vacuum, then through a sterile Seitz filter.

Morphological and biochemical characteristics. Organisms capable of multiplication at pH 4.0 were subjected to a study of morphological and biochemical characteristics in order to separate them into species. The procedures and media used for the characterization of these organisms are largely those outlined in the Manual of Methods for Pure Culture Study of Bacteria *(7)*.

Motility was determined by means of the semi-solid medium recommended by Tittsler and Sandholzer *(9)* and also by observation of hanging-drop preparations. The location

of flagella on motile species was determined according to the technique recommended by Fisher and Conn *(2)*.

Catalase activity was determined by adding a 1% solution of hydrogen peroxide to 24-hour slant cultures of the organisms and observing the evolution of gas.

To demonstrate gum (Dextran) formation by strains of *Leuconostoc*, a 10% sucrose medium described by McCleskey, Faville, and Barnett *(4)* was used.

On the basis of these tests (Table 1), 6 distinct species or types were represented among those organisms studied. Of the 6 types, only 2 could be positively identified. The Gram-positive cocci are copious gum-formers and are apparently similar, if not identical, to the Type D strains of *Leuconostoc mesenteroides* isolated from cane juice by McCleskey, Faville, and Barnett *(4)*.

TABLE 1

Morphological and physiological characteristics of 6 species of bacteria isolated from fermenting orange juice and from rotten oranges

	Leuconostoc mesenteroides	*Aerobacter aerogenes*	*Lactobacillus* sp.	*Xanthomonas* sp. "A"	*Xanthomonas* sp. "B"	*Achromobacter* sp.
Shape of cell	Coccus	Rod	Rod	Rod	Rod	Rod
Gram reaction	+	−	+	−	−	−
Pigment formation	None	None	None	Yellow	Yellow	None
Capsule formation	None	None	None	None	None	None
Spores	None	None	None	None	None	None
Motility	−	−	−	+	+	−
Flagella (location)				¼ polar	¼ polar	
Gelatin hydrolysis	−	−	−	+	+	−
Casein hydrolysis	−	−	−	−	−	−
Starch hydrolysis	−	+	−	+	+	+
Indol production	−	−	−	−	−	−
Methyl red	−	−	+	−	+	−
Voges-Proskauer	−	+	−	−	−	−
H_2S production	−	−	−	−	−	−
Nitrate reduction	−	+	−	−	+	+
Citrate utilization	−	+	−	+	+	−
Catalase production	−	+	−	+	+	+
Purple milk	A[4]	AC	A	AC	AlCP	NC
Sucrose	A	AG	A	A	A	A
Lactose	A	AG	A	A	−	−
Maltose	A	AG	A	A	A	−
Glucose	A	AG	A	A	A	A
Fructose	A	AG	A	A	A	A
Galactose	A	AG	A	A	VA	A
Mannose	A	AG	A	A	VA	A
Xylose	A	AG	−	A	A	A
Arabinose	A	AG	−	A	A	A
Mannitol	VA	AG	A	A	A	VA
Sorbitol	−	AG	A	−	−	VA
Dextrin	−	−	−	−	−	−
Starch	−	−	−	−	−	VA

[4] NC denotes no change; A, acid; C, coagulated; Al, alkaline; AG, acid and gas; P, peptonized; VA, variable acid fermentation of the sugar.

The Gram-negative, gas-forming rods correspond closely to the accepted description of *Aerobacter aerogenes* in all characteristics except the fermentation of starch and dextrin and the importance of these 2 tests is dubious.

The Gram-positive, catalase-negative rod is evidently a heterofermentative species of *Lactobacillus;* however, determination of the end-products of glucose fermentation, which was not attempted, would be necessary to completely characterize these organisms.

Both of the motile, Gram-negative bacilli form a yellow pigment on nutrient agar and are capable of liquefying gelatin. The polar location of flagella on these 2 organisms indicates that they belong in the genus *Xanthomonas,* although they do not correspond to any of the described species. Justification for placing these organisms in this particular genus is strengthened by the fact that they were most often encountered associated with pathological conditions of oranges. For example, the organism which has been designated as *Xanthomonas* sp. "B" is apparently the causal organism of the particular type of rot shown in Figure 1. Both organisms differ from *Xanthomonas citri* (Hasse) Dowson, the only species in this genus recognized as a pathogen of citrus, in several important respects. For example, *Xanthomonas citri* possesses a single polar flagellum, produces hydrogen sulfide, does not ferment glucose and, more important, produces a typical lesion on citrus fruit. On the other hand, these organisms bear a strong similarity to an organism isolated from diseased mandarin oranges by Passalacqua *(5)* and given the trinomial designation, *Bacterium citri deliciosae.*

An interesting, perhaps significant, observation in regard to these yellow-pigmented organisms was their apparent association with bacteriophage in rotten oranges. Several cultures isolated by direct streaking of juice extracted aseptically from oranges were found to be infected with phage.

The organism which has been classified as a species of *Achromobacter* does not correspond to the description of any known species in that genus. However, on the basis of the lack of pigment and motility this species apparently belongs in the genus *Achromobacter.*

Effect of pH and sugar concentration on growth of organisms. The juice of oranges will vary to a certain degree with reference to pH. Roughly this range may extend from approximately pH 3.0 to 4.0 or higher, depending upon maturity of the fruit. The arbitrary value chosen for screening these organisms, 4.0, is very nearly the upper limit for juices used in the production of frozen concentrates. To determine the lower limits of pH at which these organisms will grow in the naturally buffered orange juice, the organisms were inoculated into a series of sterile orange sera having pH values ranging from a minimum of 3.23 to a maximum of 4.0. Several filtrates having identical pH values were included because they varied slightly in regard to soluble solid content and buffer capacity. No attempt was made to alter the citric acid, sugar, or peel oil levels of the sera by the addition of these materials.

As shown in Table 2, the limiting pH for growth of these organisms in orange juice is rather sharply defined. For example, *Xanthomonas* sp. "A" grew well in 3 sera at pH 3.85, yet failed to grow at pH 3.81. The significance of these bacteria in orange juice is evidenced by the fact that 2 of the 6 types grew at pH values well within the range of the majority of juices used for the production of frozen concentrate, while 2 of the other 4 types grew at the pH of juice that would be obtained from late season fruit.

One other organism which was isolated from a rotten orange grew luxuriantly, even in the most acid serum (pH 3.23). This organism, a Gram-

TABLE 2

Effect of acidity (pH) on the growth of spoilage bacteria in orange juice

pH of filtered juice	Microorganisms					
	Leuconostoc mesenteroides	*Aerobacter aerogenes*	*Lactobacillus* sp.	*Xanthomonas* sp. "A"	*Xanthomonas* sp. "B"	*Achromobacter* sp.
3.23	—	—	—	—	—	—
3.38	—	—	—	—	—	+
3.42	—	—	—	—	—	+
3.49	—	—	—	—	—	+
3.50	—	—	—	—	—	+
3.50	—	—	—	—	—	+
3.57	+	—	—	—	—	+
3.58	+	—	—	—	—	+
3.58	+	—	—	—	—	+
3.58	+	—	—	—	—	+
3.62	+	—	—	—	—	+
3.68	+	—	—	—	—	+
3.81	+	—	—	+	—	+
3.85	+	+	—	+	—	+
3.85	+	+	—	+	—	+
3.85	+	+	—	+	—	+
3.86	+	+	—	+	—	+
4.00	+	+	+	+	+	+
4.00	+	+	+	+	+	+

negative, non-motile rod, was not included in this study since its single isolation did not indicate that it was a factor in the spoilage of any of the materials examined.

Since the pH of a 4-fold orange concentrate (42° Brix) is usually less than 0.2 of a unit lower than the raw juice from which it is made, the effect of pH of the juice upon the tolerance of these organisms to higher sugar concentrations becomes important. To demonstrate this relationship between pH and sucrose concentration, 6 batches of orange juice varying in pH from 3.23 to 4.35 and in soluble solids between 9.5 and 11.5% were filtered in the manner previously described. To aliquots of these filtrates sufficient sucrose was added to increase the soluble solids to 20°, 30°, and 40° Brix. Steaming at 100°C. for a period of 5 minutes was sufficient to kill any organisms capable of growing at the pH of the sera.

As shown in Table 3, the ability of these organisms to grow in the presence of large quantities of sugar lessened with a decrease in the pH of the juice. The *Achromobacter* sp., for example, grew in the 40° Brix serum at pH 4.35, yet would not grow in a 20° Brix juice at pH 3.58 or 3.41. Also, 3 organisms did not grow in a 40° Brix serum, even at the highest obtainable pH (4.35).

DISCUSSION

The object of this investigation has been to study some of the acid-tolerant bacteria found in citrus juices with the view that a more complete knowledge of the nature of these organisms would lead to an explanation for their occurrence in citrus juices. Of 204 bacterial cultures isolated from fermenting citrus juices and from rotting oranges, approximately ⅔ grew readily when inoculated into a sterile filtered orange juice (pH 4.0).

TABLE 3

Effect of acidity (pH) on the growth of spoilage bacteria in orange juice at varying sugar concentrations.

pH of filtered juice	Microorganisms																			
	Leuconostoc mesenteroides at °Brix				*Aerobacter aerogenes* at °Brix				*Lactobacillus* sp. at °Brix				*Xanthomonas* sp. "A" at °Brix				*Xanthomonas* sp. "B" at °Brix				*Achromobacter* sp. at °Brix			
	10*	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40
3.23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3.41	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
3.58	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
3.80	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	+	+	−	−
4.02	+	+	−	−	+	+	+	−	+	+	+	−	+	+	+	−	+	−	−	−	+	+	+	−
4.18	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+	−	−	+	+	+	−
4.35	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+

*Vary between 9.5° Brix and 11.5° Brix.

On the basis of morphological and biochemical characteristics, 5 genera were identified; namely, *Lactobacillus, Leuconostoc, Xanthomonas, Aerobacter* and, tentatively, *Achromobacter*. The 2 genera of lactic acid bacteria are perhaps the most significant, since both have been suggested as causative agents of spoilage of unpasteurized orange juice. During this study these particular organisms were, in all cases, isolated from high-count juices and not from the fruit itself. However, fruit inoculation studies now in progress indicate that these lactic acid bacteria are able to multiply rapidly within an orange with no symptoms on the exterior of the fruit to denote their presence.

The yellow-pigmented organisms which have been classified as species of the genus *Xanthomonas* were isolated mainly from rotten fruit, thus explaining the occasional appearance of large numbers of them in commercially extracted orange juice.

The isolation of *Aerobacter aerogenes* from rotten fruit is in agreement with the findings of Wolford and Berry *(12)*, who reported that *Aerobacter* species were the coliform organisms most frequently isolated from the juice of soft-rot oranges.

The tolerance of these organisms to the acidity of orange juice varies to an appreciable extent, some growing well at a pH as low as 3.4 while others do not multiply at a pH slightly below 4.0. These latter organisms, while probably not potential spoilage bacteria when considered from the standpoint of the majority of commercially-packed juices, can and do contribute materially to large microorganism counts if even a small percentage of over-mature fruit is used.

Discounting the bacteriostatic effect of the higher citric acid levels which result when citrus juices are concentrated, the pH of the juice alone has a definite effect on the ability of these organisms to proliferate in higher concentrations of sugar. For example, although none of the 6 species of bacteria isolated during this study will grow in a 40° Brix juice with a pH below approximately 4.2, 3 strains grew at this sugar concentration at

pH 4.35. However, the possibility of a pH value this high being encountered in a juice composited from many different fruit is doubtful. On the other hand, at pH 3.8, 5 of the 6 organisms grew readily in a 20° Brix juice, indicating the possibility of these organisms multiplying in single-strength juice or in the low-Brix stages of a multiple-stage evaporator of the type used for the production of frozen citrus concentrates.

SUMMARY

Six species of bacteria, representing 5 genera, have been described as the causal agents of spoilage in unpasteurized orange juice or in mature oranges on the tree. The genera represented were *Lactobacillus, Leuconostoc, Aerobacter, Xanthomonas* and, tentatively, *Achromobacter.*

All of these organisms grew rapidly in filtered orange juice at pH 4.0, while some strains were capable of growing at a considerably lower pH.

The acidity (pH) of orange juice was shown to be a factor influencing the concentration of sugar in which these organisms can grow.

LITERATURE CITED

1. Faville, L. W., Hill, E. C., and Parish, E. C. Survival of microorganisms in concentrated orange juice. *Food Technol.* **5**, 33 (1951).
2. Fisher, P. J., and Conn, J. E. A flagella staining technique for soil bacteria. *Stain Technol.*, **17**, 117 (1942).
3. Hayes, G. Personal communications (1951).
4. McCleskey, C. S., Faville, L. W., and Barnett, R. O. Characteristics of *Leuconostoc mesenteroides* from cane juice. *J. Bact.*, **54**, 697 (1947).
5. Passalacqua, T. Sul "mal della terra" dei frutti di mandarino. *Lavori Ist. Botan. Palermo*, **3**, 55 (1932).
6. Patrick, R. Bacteriological survey of some citrus canneries in Florida with special attention to *Escherichia coli*. *Proc. Florida State Hort. Soc.*, **60**, 36 (1947).
7. Society of American Bacteriologists. *Manual of Methods for Pure Culture Study of Bacteria.* Leaflets 5 and 6 (1936).
8. Teunisson, D. J., and Hall, H. H. Study of bacteria from citrus processing operations in relation to orange juice quality. *Fruit Products J.*, **26**, 199 (1947).
9. Tittsler, R. P., and Sandholzer, L. A. The use of semi-solid agar for the detection of bacterial motility. *J. Bact.*, **31**, 575 (1936).
10. Wolford, E. R. Bacteriological studies on frozen orange juice stored at −10°F. *Food Technol.*, **4**, 241 (1950).
11. Wolford, E. R., and Berry, J. A. Condition of oranges as affecting bacterial content of frozen juice with emphasis on coliform organisms. *Food Research*, **13**, 172 (1948).
12. Wolford, E. R., and Berry, J. A. Bacteriology of slime in a citrus-processing plant, with special reference to coliforms. *Food Research*, **13**, 340 (1948).

THE KINETICS OF THE OXIDATION OF STRAWBERRY ANTHOCYANIN BY HYDROGEN PEROXIDE[a]

ERNEST SONDHEIMER AND Z. I. KERTESZ
New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y.

(Manuscript received November 23, 1951)

The loss of anthocyanin is one of the major factors contributing to the color deterioration of many highly colored fruit products. A number of reactions may be envisioned by which anthocyanins are lost under conditions prevailing during the manufacture and storage of strawberry products *(10)*. Of these, the reaction of anthocyanins with hydrogen peroxide is of particular interest. Karrer, Widmer, Helfenstein, Hürliman, Nievergelt, and Monsarrat-Thoms *(12)*, and Karrer and de Meuron *(11)* showed that anthocyanins and anthocyanidins isolated from natural sources are readily oxidized by hydrogen peroxide at room temperature to discolored or colorless products. In their experiments, a large excess of hydrogen peroxide was employed. Dilthey and Höschen *(6)* presented evidence indicating that a hydroperoxide serves as an intermediate in these and similar oxidations. Quint and Dilthey *(16)* treated 9-phenyl 1,2-7,8-dibenzoxanthylium perchlorate with hydrogen peroxide and obtained a stable compound which exhibited all of the expected properties of a hydroperoxide. Although Dilthey and Höschen *(6)* have not been able to isolate a hydroperoxide from compounds closely related to naturally occurring anthocyanins, this does not detract from the plausibility of their theory that the hydroperoxides serve as intermediates. It has been noted by Leffler *(15)*, that the hydroperoxides of the naturally occurring anthocyanins give rearranged products due to cleavage of a neighboring carbon-carbon bond.

The transient presence of hydrogen peroxide in strawberry products is a distinct possibility and therefore the loss of some of the strawberry anthocyanin pigments through reaction with this hydrogen peroxide is a reasonable assumption. There are a number of reactions which may give rise to hydrogen peroxide in strawberry products. It is known, for instance, that in the presence of oxygen and cupric ions the oxidation of ascorbic acid to dehydroascorbic acid is accompanied by the formation of hydrogen peroxide *(5, 21)*. The ascorbic acid content of strawberries is comparatively high, 53-107 mg. per 100 g. *(8)*. Oxalic acid, known to be present in strawberries *(13)* may also give rise to hydrogen peroxide *(1, 9)*.

The present investigation of the kinetics of the reaction between pelargonidin 3-glucoside, an anthocyanin isolated from strawberries, and hydrogen peroxide was undertaken in order to explore the course of this reaction. Further, it seemed desirable to study and evaluate the possible

[a]Approved as Journal Paper No. 878, New York State Agricultural Experiment Station, Geneva.

contribution which such a reaction could make to the total extent of anthocyanin destruction during the processing and storage of strawberry products. Experiments were conducted in systems where initial concentrations of each constituent were known, and in strawberry juice to which hydrogen peroxide had been added.

EXPERIMENTAL

Materials. The anthocyanin used was isolated from Dresden strawberry juice by the method previously described *(22)* except that the pigment was recrystallized in the form of the picrate, rather than the chloride. For the experiments described here the anthocyanin picrate was recrystallized 6 times from hot dilute aqueous picric acid solution, converted to the chloride, and used without further purification.

In a previous publication *(23)*, we have expressed some doubt concerning the purity of anthocyanin isolated from strawberries and of the anthocyanidin obtained from it. For lack of authentic samples, it was difficult to exclude the possibility of contamination with some closely related anthocyanidin derivative. In order to obtain information on this point, pelargonidin chloride was synthesized by the method of Robertson, Robinson, and Sigiura *(17)*. The absorption spectra of the natural and synthetic anthocyanidins were identical.

The strawberry juice used in these experiments was expressed from Dresden strawberries, grown during the 1950 season at Geneva, N. Y. A small hand press was used to express the juice which was collected in large Pyrex cylinders, thoroughly mixed, and stored in gallon containers at −18°C. without any heat treatment. Using the method of Saywell and Cunningham *(19)* except for applying a spectrophotometric calibration curve at 510 mμ rather than using color standards, the iron content of the sample of strawberry juice used was found to be 0.048 millimoles per liter (2.7 p. p. m.). The ascorbic acid content *(18)* was found to be 1.7 and 2.3 millimoles per liter (29.9 and 40.5 mg. per 100 ml.) in 2 batches of juice used in the tests. The anthocyanin concentration in the juice *(22)* was found to be 0.47 millimoles per liter or 0.023%. The pH of the juice was 3.4.

J. T. Baker Chemical Company C. P. grade chemicals were used in these studies. Eastman Kodak Company ascorbic acid was employed without any further purification. The 30% hydrogen peroxide used did not contain any preservatives. Glass-distilled water was used throughout.

Procedures. In the rate studies to be reported here, only the loss of anthocyanin with time was followed. The anthocyanin concentration was obtained by measuring the optical density, log I_o/I, at 500 mμ of an appropriately diluted solution using Sorensen's disodium citrate buffer of pH 3.40. A Beckman spectrophotometer, Model B, was employed.

For the rate studies a constant temperature bath was placed within easy reach of the spectrophotometer. A convenient volume of the reaction mixture, 10 or 24 ml., without the hydrogen peroxide was allowed to reach bath temperature. Then 1 ml. of the diluted hydrogen peroxide solution was added rapidly and the solution stirred or shaken. The 1-ml. samples were removed periodically, added to 10 ml. or 20 ml. buffer of pH 3.40 and the optical density of the solution determined immediately. Less than 1 minute elapsed between the removal of the sample from the constant temperature bath and the completion of the spectrophotometric reading.

Although quenching by this method is not complete, the reaction rate is sufficiently slowed down to permit accurate measurements. For example, for the measurements at 40°C. the average error made by assuming that the reaction was completely stopped at the moment when the sample was removed from the constant temperature bath did not exceed 0.2%, while the maximum error was less than 0.7%. The reaction was followed until at least 75% of the anthocyanin was destroyed. No precautions were taken to control the amount of light reaching the reaction mixture. The half-time periods and the order of reactions were determined graphically. The reaction rate constants were calculated from the period of the half-life or from the integrated rate equations.

RESULTS

Evidence has been found for the existence of 2 mechanisms by which anthocyanin may be decolorized with hydrogen peroxide. One of these reactions is catalyzed by ferrous ions.

The Anthocyanin-Hydrogen Peroxide Reaction in Mixtures Containing Known Constituents

The non-catalyzed reaction. Clear-cut rate curves are obtained when the anthocyanin, dissolved in 0.05 *M.* disodium citrate-hydrochloric acid buffer of pH 3.4 is treated with hydrogen peroxide. As shown in Table 1, when the anthocyanin and hydrogen peroxide are present in nearly

TABLE 1

Effect of changes in the anthocyanin and hydrogen peroxide concentration at a pH of 3.40 (0.05 M. citrate buffer) and at 40°C. on the reaction rate constants

Initial anthocyanin concentration	Initial hydrogen peroxide concentration	Half-life	Order of the reaction	Rate constants
millimoles per liter	*millimoles per liter*	*minutes*		*minutes*$^{-1}$
0.488	6.79	43.0	First	0.0161
0.457	6.79	45.0	First	0.0154
0.229	6.79	48.0	First	0.0144
0.114	6.79	48.2	First	0.0144
0.453	85.4	3.5	First	0.1980
0.453	42.7	6.4	First	0.1080
0.453	28.5	11.4	First	0.0608
0.453	17.1	20.0	First	0.0347
0.453	10.7	30.5	First	0.0227
5.04	5.09	84.5	Second	0.00229[b]

[b] Minutes^{-1} (millimoles per liter)$^{-1}$.

equimolecular amounts, the reaction is second order, with excess hydrogen peroxide the half-life period is independent of the initial anthocyanin concentration and therefore the reaction is pseudo-first order. Upon plotting the pseudo-first order rate constants against the hydrogen peroxide concentration as shown in Figure 1, a straight line transecting the origin is obtained. The data summarized in Table 1 are for experiments conducted at 40°C. but similar results were obtained at 30°C. and 20°C., the average second order rate constants for the latter 2 temperatures being $1.23 \times 10^{-3} \pm 0.04$ and $0.627 \times 10^{-3} \pm 0.04$ minutes^{-1} (millimoles per liter)$^{-1}$ respectively. The calculated energy of activation is 11.3 kcal. per mole. Indications are that similar results are obtained when the anthocyanin and hydrogen peroxide are dissolved in dilute hydrochloric acid of pH 3.4 instead of the 0.05 *M.* citrate buffer of pH 3.4.

The iron-catalyzed reaction. A second mechanism was found by which anthocyanin may be decolorized by hydrogen peroxide. For a given hydrogen peroxide concentration the rate of anthocyanin destruction by this reaction is usually much faster than by the non-catalyzed path and therefore measurements were made only at 20°C. It was found that strawberry anthocyanin dissolved in dilute hydrochloric acid of pH 3.4, reacted with

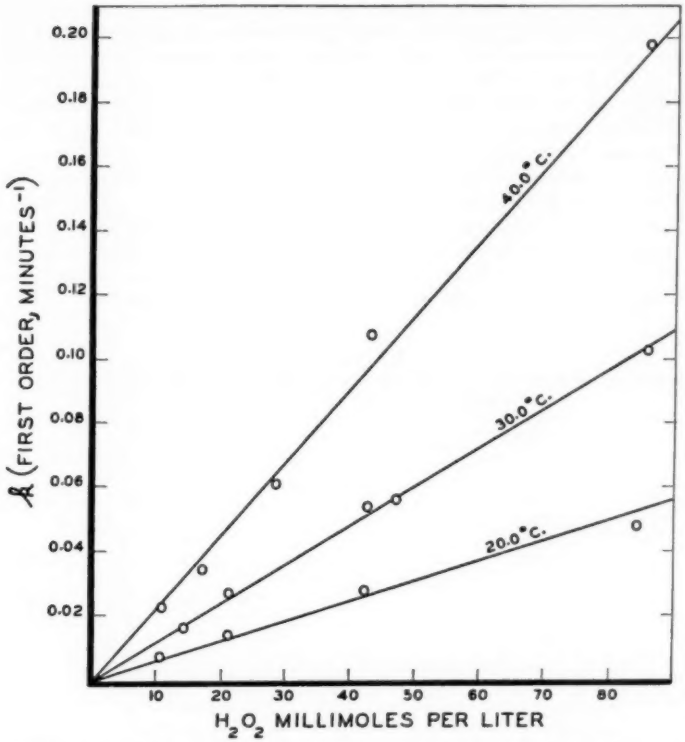


Figure 1. Effect of hydrogen peroxide concentration on the first order rate constants of the anthocyanin-hydrogen peroxide reaction in citrate buffer pH 3.4.

TABLE 2

Effect of ferrous ion and ascorbic acid on the half-life period of the anthocyanin-hydrogen peroxide reaction in dilute hydrochloric acid [c]

	Anthocyanin	Ascorbic acid	Ferrous ions	Hydrogen peroxide	Half-life
	Concentrations in millimoles per liter				
1	0.53	0	0	0	>>100 hours
2	0.53	0	0.013	0	>>100 hours
3	0.53	5.7	0	0	>100 hours
4	0.53	5.7	0.013	0	>100 hours
5	0.53	0	0	7.0	197 minutes
6	0.53	5.7	0	7.0	128 minutes
7	0.53	0	0.013	7.0	76 minutes
8	0.53	5.7	0.013	7.0	12 minutes

[c] The initial pH of the solutions was 3.40, temperature was 19.5°C.

hydrogen peroxide in the presence of ascorbic acid and catalytic amount of ferrous ions at a much more rapid rate than in the absence of either ferrous ions and/or ascorbic acid. Results of experiments illustrating this behavior are shown in Table 2. Contrary to the non-catalyzed reaction the rate of the anthocyanin destruction by the ferrous ion catalyzed reaction is decreased in the presence of citrate ion. This effect is not due to any pH changes. Some results are summarized in Table 3.

In this report the data for the above reaction are given only as the half-life periods since insufficient information is available to assign definite reaction orders and calculate the rate constants. The formation of off-colors, which may interfere in the anthocyanin determination, is observed under certain conditions and therefore in the experiments reported here conditions were chosen where this off-color development was very low.

TABLE 3

The effect of citrate ion on the half-life period of the anthocyanin-hydrogen peroxide reaction at pH 3.4 and 20°C.

Citrate ion concentration	Half-life
millimoles per liter	*minutes*
0.0	8
1.25	17
2.50	18
5.44	22
9.95	26
20.2	33
29.6	40
39.5	46
49.4	54

Initial anthocyanin concentration: 0.408 millimoles per liter.
Initial hydrogen peroxide concentration: 7.0 millimoles per liter.
Initial ascorbic acid concentration: 5.7 millimoles per liter.
Initial ferrous ion concentration: 0.013 millimoles per liter.

The Anthocyanin-Hydrogen Peroxide Reaction in Strawberry Juice

The addition of hydrogen peroxide to strawberry juice causes a decrease in the red color by a reaction which has the characteristics of the ferrous ion catalyzed anthocyanin-hydrogen peroxide reaction in the artificial mixtures. The possibility exists that the reaction of anthocyanin and hydrogen peroxide observed in strawberry juice, is the sum of contributions from the two reaction paths discussed previously. Heating the juice to 90°C. for 5 minutes prior to the rate determination at 20°C. had no effect on the rate of anthocyanin destruction, indicating that the reaction is not catalyzed by heat-labile substances as enzymes, for instance.

When the ferrous ion and ascorbic acid contents of strawberry juice were increased, an acceleration in the rate of anthocyanin-hydrogen peroxide reaction was observed. The addition of ammonium hydrogen fluoride (adjusted to pH 3.4) had a retarding effect. Examples of these observations are recorded in Table 4.

As is shown in Figure 2, a plot of the logarithm of the anthocyanin concentration in strawberry juice indicates the presence of an induction

TABLE 4

The effect of the addition of ferrous and fluoride ions and ascorbic acid on the half-life period of the anthocyanin-hydrogen peroxide reaction in strawberry juice at 20°C. and pH 3.4 [d]

Ferrous ion concentration	Ammonium bifluoride added	Ascorbic acid concentration	Half-life
millimoles per liter	*millimoles per liter*	*millimoles per liter*	*minutes*
0.048[e]	0	1.7	14
0.098[f]	0	1.7	9
0.548[f]	0	1.7	5
5.048[f]	0	1.7	1
0.048	8.3	1.7	29
0.048	4.15	1.7	19
0.048	0	0.9	21
0.048	0	3.7	5

[d] Initial hydrogen peroxide concentration in all samples was 6.25 millimoles per liter.
[e] Iron content of strawberry juice as determined by the method of Saywell and Cunningham (*19*).
[f] Iron was added as ferrous ammonium sulfate hexahydrate.

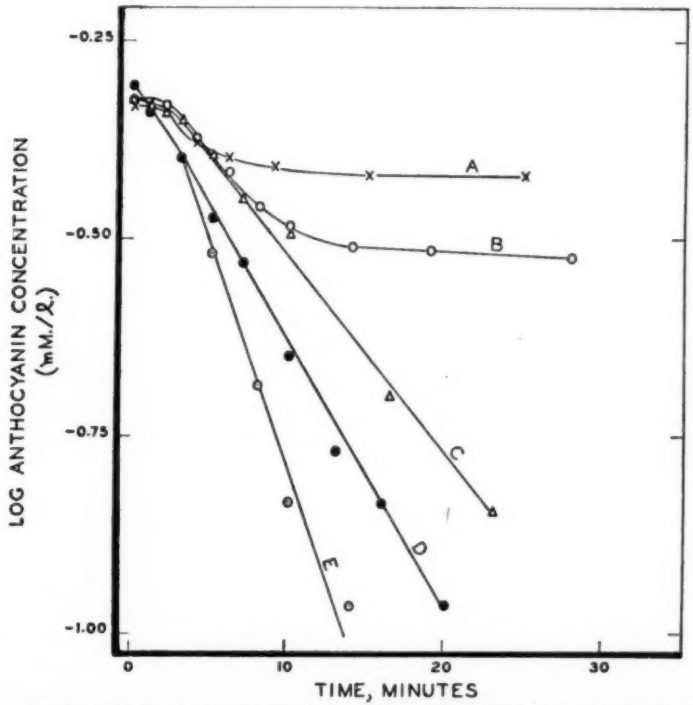


Figure 2. Rates of the anthocyanin-hydrogen peroxide reaction in strawberry juice of pH 3.4. Ascorbic acid and iron determinations showed the presence of 2.3 and 0.048 millimoles per liter respectively. The initial hydrogen peroxide concentration of A was 0.387; of B, 0.774; of C, 2.42; of D, 10.7; and of E, 77.4 millimoles per liter.

period and therefore the lines are not straight. Furthermore, the rate of the anthocyanin loss decreases only slightly as the initial hydrogen peroxide concentrations are lowered from 77.4 to 2.42 millimoles per liter. Thus the half-life period at a hydrogen peroxide concentration of 77.4 millimoles per liter was 6 minutes, at 10.7 millimoles per liter 9 minutes, and at 2.42 millimoles per liter 13 minutes.

When the citrate ion concentration of a different batch of strawberry juice was increased by 0.71%, keeping the pH constant, a decrease in the half-life period of 39% was observed.

As is shown in Figure 3, when the molecular ratio of hydrogen peroxide to anthocyanin is 2 to 1 or less, a very significant leveling off in the

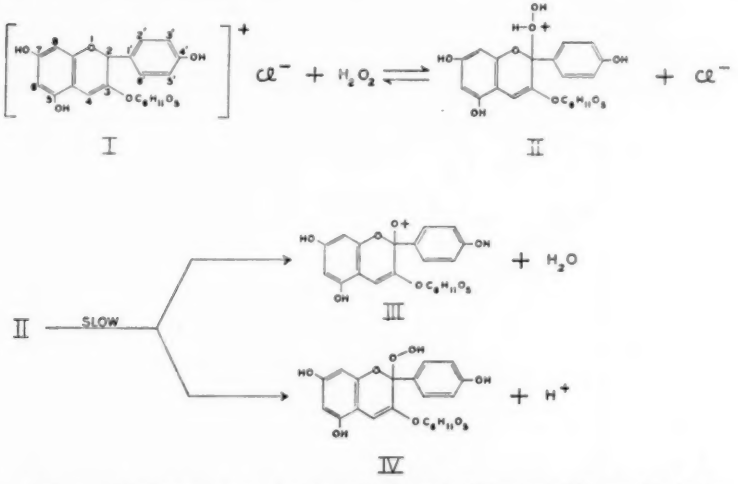


Figure 3. Suggested scheme for the anthocyanin-hydrogen peroxide reaction in the absence of iron catalysis: (I) Pelargonidin 3-monoglucoside chloride; (II) hydrogen peroxide-anthocyanin adduct; (III) oxygen cationic intermediate; and (IV) pelargonidin 3-monoglucoside, 2-hydroperoxide. Compounds (III) and (IV) are believed to yield identical rearranged products.

rate of anthocyanin destruction occurs before more than a fraction of the anthocyanin is destroyed. Thus, at an anthocyanin concentration of 0.4 millimoles per liter and a hydrogen peroxide concentration of 0.774 millimoles per liter (0.0026%) approximately 40% of the pigment is destroyed in 20 minutes. On lowering the hydrogen peroxide concentration to 0.387 millimoles per liter (0.0013%) about 20% anthocyanin pigment was destroyed in 20 minutes. Two explanations may be offered to account for this behavior. First, there may be a competition for the hydrogen peroxide between anthocyanin and other constituents of the system. Second, the destruction of anthocyanin in strawberry juice may involve more than one equivalent of hydrogen peroxide.

DISCUSSION

The reaction not catalyzed by ferrous ions. One possible mechanism for the reaction which is consistent with the kinetic data as well as with the conclusions reached from degradation studies by other workers is presented in Figure 3. In this scheme the hydrogen peroxide addition is represented as occurring through the carbonium ion of the anthocyanin (I). This is in accordance with the conclusions of Dilthey and coworkers *(6, 7, 16)* but has not been confirmed kinetically. Karrer and coworkers *(11, 12)* showed that when malvin chloride, 3,5,7,4′-tetrahydroxy-3′,5′-dimethoxy-3,5-diglucosidoflavylium chloride, is oxidized with hydrogen peroxide malvone is obtained in 65% yield. Malvone is believed to have the structure shown in Figure 4. The formation of this oxidation product is compatable with

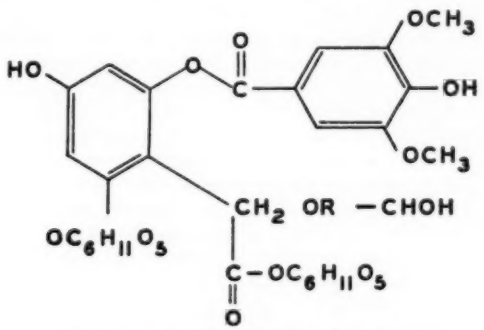


Figure 4. Structural formula of malvone.

hydrogen peroxide addition in position 2 of the flavylium salt. On the basis of these results, the addition of hydrogen peroxide to pelargonidin 3-glucoside (I) is shown in Figure 3 as occurring in position 2 also. The oxidation products of pelargonidin 3-glucoside are now being investigated. It is hoped that these studies will provide direct information on the site of peroxidation. Although the mechanism for the oxidation as pictured here represents the addition of hydrogen peroxide and anthocyanin as a reversible step followed by a slow decomposition of the addition product, the kinetical data do not exclude the possibility that the hydrogen peroxide addition to the pigment is the rate-determining step.

The iron-catalyzed reaction. The work reported here indicates that in systems containing ascorbic acid, the oxidation of strawberry anthocyanin by hydrogen peroxide is greatly accelerated by small amounts of ferrous ions. It is further shown in Table 3 that fluoride ions cause a decrease in the oxidation rate. In an article on the effect of amorphous ferric hydroxide on the rate of oxidation by hydrogen peroxide of certain dyes, Krause and Polanski *(14)* discuss the behavior of ferrous ions in related systems. They state that acceleration of the oxidation rate is observed only with ferrous ions and that ferric ions are incapable of increasing the rate if the formation of ferric hydroxide is prevented.

Recent work on the low temperature polymerization of rubber indicates an interesting similarity of several aspects of these systems to the oxidation described here. The polymerization rate of certain monomers to rubber at low temperatures depends upon the availability of free radical chain initiators. Wall and Swoboda *(25)* discuss an effective two-phase system for bringing about such low temperature polymerization. According to these authors, the important constituents of the "rubber recipe" which cause chain initiation are hydrogen peroxide, ferrous and phosphate ions and sorbose. They explain the usefulness of the "recipe" by the assumption that the purpose is to develop a low but steady ferrous ion concentration. Sorbose is believed to function as a reducing agent for the ferric ions which form during the course of the reaction.

The function of ascorbic acid in our system is believed to be identical to that of sorbose in the above "rubber recipe." That ascorbic acid is capable of reducing ferric ions was shown by Szent-Györgyi *(24)*. In systems containing small amounts of ferrous ions, ascorbic acid is not necessary in order to obtain the rapid rate initially; but it is needed to maintain the accelerated rate. This can be seen by comparing the half-life periods of samples Nos. 7 and 8 in Table 2. In sample No. 7, the anthocyanin loss was rapid during the first few minutes and then slowed down markedly. No such change in slope was observed when ascorbic acid was also present, as in sample No. 8. Table 2 further shows that if ascorbic acid is added to an anthocyanin-hydrogen peroxide system to which no ferrous ions were added (sample No. 6), the rate of the reaction is greater than in the absence of ascorbic acid (sample No. 5). This relatively small acceleration in the rate is believed to result from the presence of trace metallic impurities capable of catalyzing the reaction similarly to ferrous ions.

The inhibitory effects of citrate and fluoride are attributed to their complexing action of ferric ions. Simon, Haufe, Reetz, and Preissler *(20)* found that the efficiency of ferric salts in catalyzing the decomposition of hydrogen peroxide depends on the nature of the anion, the fluoride being the least active of the anions tested. Bobtelsky and Simchen *(4)* and Bobtelsky and Kirson *(3)* report that citrate ions at certain concentrations had an inhibiting effect on the iron-catalyzed hydrogen peroxide decomposition. In view of these considerations, the following scheme is suggested for the ferrous ion catalyzed reaction of hydrogen peroxide with anthocyanin:

$$H_2O_2 + Fe^{++} \longrightarrow HO^{\cdot} + HO^{-} + Fe^{+++}$$
$$HO^{\cdot} + Fe^{++} \longrightarrow HO^{-} + Fe^{+++}$$
$$Fe^{+++} + \text{ascorbic acid} \longrightarrow Fe^{++} + \text{dehydroascorbic acid}$$
$$HO^{\cdot} + \text{anthocyanin} \longrightarrow ? \longrightarrow \text{oxidation products}$$

This series of reactions is very similar to the mechanism for the low temperature polymerization of rubber presented by Baxendale, Evans, and Park *(2)*. Since it is possible that ferric ions may be reduced during the anthocyanin-hydrogen peroxide reaction an acceleration of the rate of color loss may occur upon the addition of ferric ions to a system containing the other necessary constituents. One possible path for the reduction to ferrous ions was discussed by Simon et al. *(20)*. For this reaction the hydroperox-

ide anion, HO_2^-, appears necessary. Another possibility is that the ferric ions are reduced to ferrous ions by oxidizing one or more of the primary anthocyanin oxidation products *(26)*.

Since dehydroascorbic acid is formed during the ferrous ion-catalyzed oxidation, a number of experiments were conducted to ascertain whether this compound is the actual oxidizing agent. These indicated that dehydroascorbic acid is not capable of oxidizing the anthocyanin at a rate which would account for the velocity of the reaction.

The free radical reaction may proceed by a somewhat different path. If the anthocyanin hydroperoxide, IV, Figure 3, splits off a hydroxyl radical, a free radical with the identical atomic structure as III, Figure 3, will result. This then can rearrange to unidentified products.

SUMMARY

1. The kinetics of the reaction of an anthocyanin preparation from strawberries with hydrogen peroxide have been studied. Evidence for two reaction paths is presented and mechanisms for the reactions are discussed. One of these reactions is catalyzed by ferrous ions.

2. The reaction leading to color loss has also been studied in strawberry juice to which hydrogen peroxide has been added. Although the formation of hydrogen peroxide in strawberry juice has not been demonstrated, it has been shown that, if present, hydrogen peroxide could be responsible for some of the color loss in strawberry products.

LITERATURE CITED

1. Baczko, v., K., and Schröer, E. The oxidation of oxalic acid solution by elementary oxygen in the presence of manganese. *Z. Electrochem.*, **47**, 801 (1941).
2. Baxendale, J. H., Evans, M. G., and Park, G. S. Mechanism and kinetics of the initiation of polymerization by systems containing hydrogen peroxide. *Trans. Faraday Soc.*, **42**, 155 (1946).
3. Bobtelsky, M., and Kirson, B. Catalytic decomposition of hydrogen peroxide in the presence of ferric citrate. *Compt. rend.*, **208**, 1577 (1939).
4. Bobtelsky, M., and Simchen, A. E. Solutions of citric and ferric ions with and without addition of hydrogen peroxide. *Compt. rend.*, **208**, 1646 (1939).
5. Dekker, A. O., and Dickinson, R. G. Oxidation of ascorbic acid by oxygen with cupric ion as catalyst. *J. Am. Chem. Soc.*, **62**, 2165 (1940).
6. Dilthey, W., and Höschen, W., Pyrenium salts (XX). Oxidation of pyrenium salts. *J. prakt. Chem.*, **138**, 42 (1933).
7. Dilthey, W., and Quint, F. Pyrenium compounds. XVIII. Oxidation of pyrenium salts. *J. prakt. Chem.*, **131**, 1 (1931).
8. Hansen, E., and Waldo, G. F. Ascorbic acid content of small fruits. *Food Research*, **9**, 453 (1944).
9. Huelin, F. E. Use of oxalic acid in the determination of ascorbic acid. *Analyst*, **75**, 391 (1950).
10. Joslyn, M. A. Color retention in fruit products. *Ind. Eng. Chem.*, **33**, 308 (1941).
11. Karrer, P., and de Meuron, G. Plant coloring matter (XL). Decomposition of anthocyanins by oxidation. Constitution of Malvone. *Helv. Chim. Acta*, **15**, 507 (1932).
12. Karrer, P., Widmer, R., Helfenstein, A., Hürliman, W. Nievergelt, O., and Monsarrat-Thoms, P. Plant coloring matters (IV). Anthocyanins and anthocyanidins. *Helv. Chim. Acta*, **10**, 729 (1927).
13. Kohman, E. F. Oxalic acid in foods and its behavior and fate in the diet. *J. Nutrition*, **18**, 233 (1939).
14. Krause, A., and Polanski, P. Oxidation of aqueous solutions of organic dyes with hydrogen peroxide in the presence of amorphous ferric hydroxide as a catalyst in a heterogeneous system. *Ber.*, **71**, 1763 (1938).
15. Leffler, J. E. Cleavages and rearrangements involving oxygen radicals and cations. *Chem. Rev.*, **45**, 385 (1949).
16. Quint, F., and Dilthey, W. Pyrenium salts (XIV). Oxidation of pyrenium salts. *Ber.*, **64**, 2082 (1931).

17. ROBERTSON, A., ROBINSON, R., AND SIGIURA, J. Synthesis of pelargonidin chloride by means of O-benzoylphloroglucinaldehyde. *J. Chem. Soc.*, (1928) 1533.

18. ROBINSON, W. B., AND STOTZ, E. The indophenol-xylene extraction method for ascorbic acid and modifications for interfering substances. *J. Biol. Chem.*, **160**, 217 (1945).

19. SAYWELL, L. G., AND CUNNINGHAM, B. B. Determination of iron. *Ind. Eng. Chem., Anal. Ed.*, **9**, 67 (1937).

20. SIMON, A., HAUFE, W., REETZ, T., AND PREISSLER, R. Active iron (VII). Ferric salt catalysts. *Z. anorg. u. allgem. Chem.*, **230**, 129 (1936).

21. SILVERBLATT, E., ROBINSON, A. L., AND KING, C. B. Kinetics of the reaction between ascorbic acid and oxygen in the presence of copper ion. *J. Am. Chem. Soc.*, **65**, 137 (1943).

22. SONDHEIMER, E., AND KERTESZ, Z. I. Anthocyanin pigments. Colorimetric determination in strawberries and strawberry products. *Anal. Chem.*, **20**, 245 (1948).

23. SONDHEIMER, E., AND KERTESZ, Z. I. The anthocyanin of strawberries. *J. Am. Chem. Soc.*, **70**, 3476 (1948).

24. SZENT-GYÖRGYI, A. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. Description of a new carbohydrate derivative. *Biochem. J.*, **22**, 1402 (1928).

25. WALL, F. T., AND SWOBODA, T. J. An oxidation-reduction cycle in emulsion polymerization systems. *J. Am. Chem. Soc.*, **71**, 919 (1949).

26. WIELAND, H., AND FRANKE, W. Mechanism of the oxidation process. XII. The activation of hydrogen peroxide by iron. *Ann.*, **457**, 1 (1927).

FACTORS WHICH AFFECT THE DRAINED WEIGHT AND OTHER CHARACTERISTICS OF HEAT-PROCESSED RED CHERRIES[a]

R. T. WHITTENBERGER
Eastern Regional Research Laboratory,[b] Philadelphia, Pennsylvania

(Manuscript received February 2, 1952)

Despite the importance of drained weight in processed red cherries, knowledge concerning the factors which govern drained weight remains meager. Variations in drained weight commonly are attributed either to differences in growth conditions of the cherries or to variations in spray treatments. That other factors, however, may affect drained weight is shown by the results of the present study.

The study was conducted on a laboratory scale and the experiments were designed principally to investigate the causes for the variations in drained weight rather than to establish new procedures which might be applicable directly to commercial practice. The study showed that the drained weight of cherries might be raised or lowered by different preprocessing and processing treatments.

MATERIALS AND METHODS

In 1950, 1 lot of Montmorency cherries was picked from each of 2 orchards near Orrtanna, Pennsylvania. Lot A was picked very carefully with stems attached to the fruit; Lot B was bruised slightly in being picked without stems. About 8 hours after being picked, the cherries were placed in storage maintained at 2°C.(35°F.) and 90% relative humidity. They remained sound for a long period and served as uniform raw material for the experiments.

A rapid laboratory method for processing the cherries was devised. In each test 40 dry, uniform cherries were pitted with a portable pitter at a predetermined temperature [2°, 10°, or 24°C.(35°, 50°, or 75°F.)]. Each cherry was permitted to drain on a dry table top for about 3 minutes before being pooled with the other cherries and weighed. The cherries were then poured into a beaker containing an equal weight of boiling distilled water, brought to a boil, and boiled gently for 1 minute. Water lost through evaporation was replaced. Drained weight (cherries drained on an 8-mesh screen for 2 minutes) was measured after the cherries had stood for 24 hours. The results obtained by this method correlated satisfactorily with those of a standard method of canning.

Soluble solids were estimated with an Abbé refractometer, and firmness was measured with a pressure tester *(6)*. The color of the cooked filtered juice was indicated by the percent transmittance in a 1-cm. cell, measured with a spectrophotometer, at a wavelength of 520 mμ. In some experiments cherries were bruised by being dropped 3 times from a height of 3 feet into a porcelain tray. The extent of this bruising approximated that observed in some commercially handled cherries.

RESULTS

Changes in cherries during storage. For the sake of brevity, only a few of the data are presented in this report. Despite shrinkage of the fresh cherries, the drained weight in grams of processed fruit recoverable from

[a]Report of study made under the Research and Marketing Act of 1946.
[b]One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

a constant number of fresh cherries increased steadily with the period of storage (Table 1). The increase was greater in the A than in the B cherries, whose original drained weight was relatively high. Meanwhile the cherries increased in soluble solids (percentage basis) and in firmness, showed almost no change in pitting losses (percentage basis), but decreased somewhat in acidity and in red color.

Drained weight as used here indicates the overall yield of processed cherries, since the drained weight is related directly to the weight of the original fresh cherries. On the other hand, the commonly used ratio of drained weight to freshly pitted weight does not indicate reliably the overall yield (observe columns 2 through 4 of Tables 1 and 2). The ratio takes into account neither the differences in pitting losses among various samples nor the changes in weight of cherries which occur between harvest and time of pitting. During this period cherries may either gain or lose weight.

Spoilage of the fresh cherries was negligible for the first 3 or 4 weeks, although after 8 weeks of storage about 10% of the B cherries were culls. Spoilage in lot A (stems attached) did not begin until after 7 to 8 weeks of storage. The cherries were not washed or given any special treatment before storage. Commercially handled fruit, however, probably would not remain sound if stored for 3 weeks at 2°C.

Increasing the drained weight. A number of treatments other than prolonged cold storage were found to result in an increase in the drained weight of the lot A cherries (Table 2). After the cherries had been in cold storage for 1 week, bruising them and permitting them to stand or to soak for several hours before being processed increased their drained weight. The bruising had little effect on the color of the processed cherries, although if bruised cherries were stored for more than about 24 hours at room temperature (24°C.), their color deteriorated. They remained relatively stable, however, if held at a low temperature (2°C.) in water or in a dilute calcium chloride solution. At the low temperature their pitting losses were minimized. The bruised cherries were less firm in the raw state but more firm in the processed state than were unbruised cherries.

After the fresh cherries (Lot A) had been stored for 60 days at 2°C. and their resultant drained weight had increased to a relatively high value, bruising had almost no effect on drained weight. Similar results were obtained with the cherries of Lot B (high drained weight) after a storage period of 16 days. It is believed that the slight bruising of the B cherries attendant with their picking was responsible in part for their high drained weight and for their lack of response to additional bruising.

Further studies were conducted in 1951 on 5 lots of cherries picked very carefully both with and without attached stems. All 5 lots initially had low drained weights and responded to bruising in a manner identical with that of Lot A. Another 2 lots, bruised slightly while being picked, had drained weights of intermediate value. The final 3 lots studied in 1951 were commercially handled cherries which were rather severely bruised. Their drained weights were uniformly high.

There were conditions, however, under which the bruising of cherries resulted in a lowering of the drained weight. In 1951, for instance, the bruising of carefully handled cherries just a few minutes before they were pitted and processed lowered their drained weight from 120.0 to 115.6 g. (average of 6 experiments with 3 lots of cherries). The bruising caused excessive loss of juice and tearing of cherries during the pitting operation. On the other hand, the drained weight of similarly bruised cherries which were stored in air or soaked in water for several hours after being bruised was 129.6 g. In addition, the loss from their pitting was comparatively small.

TABLE 1

Effect of storage in air at 2°C. on drained weight and other characteristics of red cherries

Days stored	Weight of 40 fresh whole cherries	Drained weight of 40 processed pitted cherries	Ratio of drained weight to freshly pitted weight	Pitting loss	Soluble solids in fresh juice	pH of fresh juice	Compression of fresh cherries (firmness)	Color, transmittance at 520 mμ of processed juice
	g.	*g.*		%	%		%	%
Lot A								
5	175	104	0.69	14.0	13.0	3.5	24.4	10
12	174	105	0.71	14.4	13.5	3.5	.....	10
26	172	108	0.75	.....	13.8	3.6	.....	12
40	169	119	0.83	14.7	13.8	3.7	23.6	....
59	166	120	0.85	15.0	13.8	3.8	23.1	22
76	163	127	0.92	15.0	13.8	4.0	22.1	27
101	160	145	1.07	14.3	14.4	4.3	17.7	39
Lot B								
12	168	118	0.89	20.0	13.8	3.7	21.1	12
24	165	117	0.88	20.1	14.0	3.8	20.2	11
60	155	124	0.99	18.6	14.3	4.0	17.9	18
74	152	129	1.03	18.4	14.3	3.9	16.0	40

TABLE 2

Effect of various treatments, including bruising and soaking in calcium chloride solution, on drained weight and other characteristics of red cherries, Lot A

Treatment	Weight of[c] 40 fresh whole cherries	Drained weight of 40 pitted processed cherries	Ratio of drained weight to freshly pitted weight	Pitting loss	Soluble solids in fresh juice	Compression of fresh cherries (firmness)	Color, transmittance at 520 mμ of processed juice
	g.	*g.*		%	%	%	%
Original cherries, 2°C.	175	101	0.67	13.9	13.3	22	10
Soaked in water for 24 hours at 2°C.	186	102	.....	.....	12.2	21	10
Stored in air for 24 hours at 24°C.	171	106	0.73	15.3	14.0	22	10
Bruised, stored in air for 24 hours at 24°C.	158	117	0.88	16.1	14.0	33	12
Soaked in 0.2% $CaCl_2$ for 24 hours at 24°C.	187	113	0.74	17.3	11.7	20	17
Bruised, soaked in 0.2% $CaCl_2$ for 24 hours at 24°C.	162	121	0.89	16.5	11.6	30	15
Soaked in 0.2% $CaCl_2$ for 24 hours at 2°C.	184	115	0.75	15.4	12.7	19	13
Bruised, soaked in 0.2% $CaCl_2$ for 24 hours at 2°C.	172	121	0.83	14.8	12.8	33	13

[c] The original weight of the 40 cherries used for each treatment was 175 g. Before treatment, all samples had been stored at 2°C. for 1 week.

It is apparent that the effects of bruising on drained weight depend partly on the extent of bruising, the time of bruising, the temperature and duration of the storage period after bruising, and the condition of the cherries at the time of bruising. Under the experimental conditions employed by Hills, Whittenberger, Robertson, and Case *(3)*, bruising had no effect on the yield of final product. The bruising did, however, increase both the tenderometer reading and the ratio of drained weight to put-in weight of the canned cherries.

Another means of increasing the drained weight of the cherries (both Lots A and B) was to store them in air at 24°C. For instance, the storing of the Lot A cherries at this temperature for 24 hours increased their drained weight from 101 to 106 g. The increase was proportional to the length of the storage period for the first 2 or 3 days. Bruised fruit, however, did not keep satisfactorily at this temperature for more than about 24 hours.

Finally, drained weight was increased by soaking and processing the cherries in a dilute solution of calcium chloride (Table 2 and Figure 1). Calcium ions were effective at both 2° and 24°C. on both bruised and unbruised fruit. The increase was greater with cherries having a low drained weight (Lot A) than with fruit of high drained weight (Lot B). Most processing plants use hard water which contains calcium for the soaking and canning of red cherries.

Decreasing the drained weight. Treatment of cherries with pectin extractants lowered their drained weight. In one experiment, processing the cherries in a dilute solution of ammonium oxalate decreased their drained weight by 33% (Figure 1). Prolonged heating had a similar effect. For example, cherries (Lot A) boiled for a few seconds, 10 minutes, and 30 minutes, had drained weights of 118, 107, and 101 g., respectively. However, after the raw cherries had become toughened by storage for 3 months, the length of the heating period had little effect on drianed weight. As

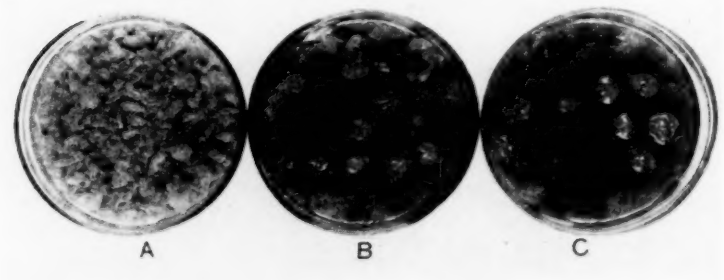


Figure 1. Effects of ammonium oxalate and calcium chloride on processed red cherries. A, Processed in 1.4% ammonium oxalate. Drained weight, 67 g. B, Processed in distilled water. Drained weight, 100 g. C, Processed in 0.5% calcium chloride. Drained weight, 115 g.

stated previously, the bruising of cherries (3 lots in 1951) lowered their drained weight if they were processed immediately after being bruised.

Miscellaneous observations on drained weight. Within each lot there was no relationship between the soluble solids content of raw cherries and the drained weight of processed fruit. In Lot A, for instance, cherries containing 11.5, 13.0, and 14.1% soluble solids had identical drained weights. Samples differing in soluble solids content were obtained by manually separating the cherries into three color groups (orange red, red, mahogany red). Similar results were obtained with the cherries in Lot B.

In some experiments cherries of both Lots A and B were separated into groups differing in total solids content by flotation of the raw cherries in brine solutions of different specific gravities. No apparent relationship between total solids content and drained weight was observed within either lot. The specific gravity method of grading, however, may be useful for predetermining factors other than drained weight. The correlation within each lot between specific gravity and soluble solids content and between specific gravity and color was good. Light colored cherries were separated quickly and conveniently from average to dark colored fruit. A minor limitation of this method of grading cherries was that the pits differed more widely in specific gravity than did the whole cherries.

Soaking the cherries (Lot A) in distilled water had little effect on the yield of final product (Table 2). Although unbruised cherries increased in weight by about 6% when soaked for 24 hours, this increase was not reflected in the final yield. The bruised cherries did not gain in weight when soaked.

Drained weight was dependent partly on the temperature of the cherries. The same cherries measured at 84°, 24°, and 4°C. (at 2-hour intervals) had drained weights of 113.8, 122.8, and 125.2 g., respectively.

The error involved in the measurement of drained weight was nearly as great as that incurred during all the other steps of sampling, pitting, and processing. For instance, the drained weight of the same 40 cherries was measured 5 times. After each measurement, the cherries and juice were recombined and permitted to come to equilibrium. In contrast with that of canned and stored cherries, the drained weight did not decrease linearly with each successive measurement, but varied in an irregular manner. The mean drained weight was 135.5 g. with a standard error of 0.41 g. On the other hand, the mean drained weight of 5 separately processed samples of the same cherries was 133.5 g. with a standard error of 0.47 g.

Comparison of methods. The results obtained by the rapid method of processing correlated well with those obtained by a standard method of canning, the coefficient of correlation between the ratios of drained weight to freshly pitted weight being +0.958 (Table 3). However, quantitative differences did exist. The rapid method was the more sensitive and magnified to a higher degree the differences in ratios of drained weight to freshly pitted weight among various samples.

The 2 sets of data of Table 3 are not strictly comparable. Although the results of the rapid method were obtained 24 hours after processing, those of the standard method were obtained after the cherries had been stored in cans for 8 months at 24°C. During storage canned cherries may undergo changes in color *(1, 2)*, acidity *(2)*, texture *(2)*, and possibly in drained weight.

DISCUSSION

The collective data of this study permit the formulation of a hypothesis concerning the nature of drained weight. High drained weight in heat-processed cherries seems to be associated with a high degree of tissue cohesiveness, which in turn is dependent on the strength or heat stability of the intercellular cement. Microscopic examination reveals that from tissues exhibiting low drained weight, single intact cells and clusters of cells may slough off of their own accord or may be separated easily with a

TABLE 3

Drained weights obtained by rapid and standard methods of processing red cherries[d]

Cherries from lot	Storage period of fresh cherries	Treatment after storage	Ratio of drained weight to freshly pitted weight	
			Rapid method	Standard method[e]
	days			
A	26	None	0.741±0.0064[f]	0.822
B	29	Soaked in water, 10°C., for 24 hours	0.809	0.838
B	29	Soaked in 30% sucrose for 24 hours	0.814	0.884
A	39	Processed at once after being pitted	0.817	0.862
A	59	None	0.853±0.0031[f]	0.878
....	....	Commercial cherries	0.860	0.857
A	39	Processed 5 hours after being pitted	0.870	0.896
A	60	Soaked in water, 2°C., for 24 hours	0.877±0.0047[f]	0.897
B	24	None	0.881±0.0059[f]	0.890
A	61	Stored at 24° and 4°C. for 1 day each	0.913	0.911
A	61	Bruised, air 24°C., water 2°C.	0.974±0.0051[f]	0.921
A	74	Stored at 24°C. for 1 day, 4°C. for 2 weeks	1.022	0.967
B	27	Stored at 24°C. for 2 days	1.048	0.963

[d] The coefficeint of correlation was +0.958.
[e] Cherries were processed to a central-can temperature of 90°C.(195°F.) in No. 2 tins.
[f] Standard error, n = 5.

microneedle (Figure 2). This indicates that the intercellular cement is comparatively weak. On the other hand, single intact cells cannot readily be separated from freshly processed tissues showing high drained weight. When a cell is subjected to tension with a microneedle, its wall fractures before the intercellular cement fails.

In the present concept, then, the processed cherry may be looked upon as a cellulose sponge whose liquid-holding capacity is dependent on the maintenance of its cellular structure. Variations in soluble solids content of cherries otherwise identical should have little effect on drained weight, since the variations would not affect the intercellular cement and the cell membranes are no longer differentially permeable after processing. Our

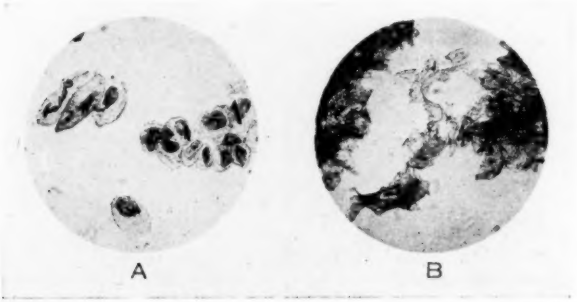


Figure 2. Photomicrographs of microdissected tissues of processed red cherries (29 ×). A, Tissues of low drained weight. Single whole cells and small clusters of cells separate readily from the main body of tissue. B, Tissues of high drained weight. Cells fracture rather than separate when subjected to tension.

studies indicate that soluble solids data alone cannot be used reliably to predetermine drained weight. As a matter of coincidence, however, there may be a rough relationship between soluble solids content and drained weight of different lots of cherries, since soluble solids content under some conditions may be related to a more fundamental factor, the pectin-cellulose structure of tissues.

It might be expected, however, that agents which affect the intercellular cement (composed principally of pectic compounds) would also affect drained weight. Such is the case. The intercellular cement is weakened and drained weight is decreased by: 1, treating tissues with pectin extractants; 2, prolonged cooking of tissues; and 3, mechanical pressure (that is, placing a weight on the cherries during the measurement of drained weight). The pressure effect is proportionally greater in cherries of low drained weight (weak intercellular cement) than it is in cherries of high drained weight (strong intercellular cement). Permanent damage is done to the tissues of the former, but tissues of the latter recover.

On the other hand, the intercellular cement of freshly picked, unbruised cherries is strengthened and drained weight is increased by: 1, bruising combined with aging of tissues; 2, physiological aging or "conditioning" of tissues; and 3, treating tissues with calcium ions.

The manner in which bruising increases the strength of the intercellular cement or its capacity to resist degradation by heat is not clear. It is known, however, that bruising induces definite physiological responses and generally increases the rate of respiration. In cherries, the bruised tissues may be partially repaired and strengthened. The aging of tissues evidently results either in the strengthening of the intercellular cement or in the thickening of cell walls, or more probably, in both. In general, young tissues are more tender, fragile, and internally flexible than are similar old tissues. Our results are in agreement with the observation that aging and its effects occur more rapidly at a high temperature (24°C.) than at a low temperature (2°C.). The effect of calcium ions on the intercellular cement is well known *(4, 5)*.

After being picked, cherries continuously undergo changes which affect their drained weight, even though they are given no experimental treatment. Of utmost importance in governing the changes are temperature of storage, duration of storage, and extent of bruising.

Unfortunately, the 3 factors listed above are difficult to control in large-scale experimental studies or in commercial practice. Pickers differ somewhat in the extent to which they bruise cherries, although they bruise nearly all samples rather severely. The picked cherries are permitted to stand for irregular periods and at various temperatures in the orchards or in trucks before being delivered to the processing plant. As a result, the principal variations in the drained weight of different lots of cherries may be caused by unnoticed differences in the manner of handling or storing the cherries rather than by differences in any other factor. In studies on the effects of maturity, spray treatments, soluble solids content, and soaking on drained weight, the different lots of cherries should be completely identical in extent of bruising and in post-harvest treatment. If the bruising and post-harvest treatments of all lots are sufficiently severe,

almost no differences in drained weights should be expected. On one hand, the treatments increase the drained weights to near maximum values; on the other hand, they cause losses in red color, acidity, tenderness, and possibly in flavor. In addition, the hardness of the processing water, which may change with the rainfall, may sometimes cause variations in drained weight.

The results presented in this report were confirmed in large measure by recent studies on 10 additional lots of cherries. The gap between the present findings and commercial practice, however, remains. Although it may be possible to account for variations in properties of processed fruit, effective control over the factors which cause the variations may be difficult.

SUMMARY

The successful storage of carefully picked and handled red cherries in air at 2°C. for 3 weeks suggested the possibility of lengthening the normally short processing season. During storage, the cherries underwent changes which increased their drained weight, firmness, and % soluble solids content. They showed almost no change in flavor and pitting losses, and decreased slightly in acidity and in red color.

From the collective data a general interpretation of the factors which affect drained weight was made. High drained weight was associated with a high degree of tissue cohesiveness, which in turn was dependent on the strength or heat stability of the intercellular cement. Microscopic studies showed that single whole cells could be separated easily from tissues of low drained weight but not from tissues of high drained weight. The processed cherry may be looked upon as a cellulose sponge whose liquid-holding capacity is dependent on the maintenance of its cellular structure. Factors which weaken the intercellular cement, such as prolonged cooking, extraction with pectin solvents, and mechanical pressure, decrease drained weight. Factors which strengthen the cement, such as post harvest aging or "conditioning" of the raw tissues, bruising combined with aging, and treatment with calcium ions, increase the drained weight.

LITERATURE CITED

1. Culpepper, C. W., and Caldwell, J. S. Behavior of anthocyan pigments in canning. *J. Agr. Research*, **35**, 107 (1927).
2. Griswold, Ruth M. Factors influencing the quality of home-canned Montmorency cherries. Mich. State Agr. Exp. Sta., *Tech. Bull.*, **194** (1944).
3. Hills, Claude H., Whittenberger, R. T., Robertson, W. F., and Case, W. H. Some effects of bruising on the yield and quality of canned Montmorency cherries. *Food Technol.*, in press.
4. Kertesz, Z. I. The effect of calcium on plant tissues. *Canner*, **88** (7), 26 (1939).
5. True, R. H. Significance of calcium in higher green plants. *Science*, **55**, 1 (1922).
6. Whittenberger, R. T., and Marshall, R. E. Measuring the firmness of red tart cherries. *Food Technol.*, **4**, 311 (1950).

DIFCO

Isolation and Cultivation of BRUCELLA

Preliminary Enrichment

Isolation of *Brucella* strains from blood specimens is best accomplished by preliminary enrichment of the sample in medium prepared from Bacto-Tryptose Broth. For isolation from samples of infected milk no enrichment is required.

Isolation of Strains

After enrichment, the blood specimens are streaked on plates of Bacto-Tryptose Agar and the plates are incubated at 37° C. Milk samples are streaked on plates of Bacto-Tryptose Agar prepared with crystal violet to inhibit the streptococci and other Gram-positive organisms.

Differentiation of Types

Bacto-Tryptose Agar to which thionin or basic fuchsin has been added is recommended for the differentiation of newly isolated strains of *Brucella*.

Mass Cultivation

Bacto-Tryptose Agar, prepared without addition of dyes or other ingredients, supports luxuriant growth of all *Brucella*. It is an excellent medium for mass cultivation of the organisms for preparation of bacterial vaccines or antigens.

Specify "DIFCO"

THE TRADE NAME OF THE PIONEERS
In the Research and Development of Bacto-Peptone and Dehydrated Culture Media

DIFCO LABORATORIES
DETROIT 1, MICHIGAN